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EFFECTS OF THERAPY ON BIOLOGY AND KINETICS OF THE RESIDUAL TUMOR, PART A: PRE-CLINICAL ASPECTS

Editors: Joseph Ragaz
Linda Simpson-Herren
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EFFECTS OF THERAPY ON BIOLOGY AND KINETICS OF THE RESIDUAL TUMOR, PART A: PRE-CLINICAL ASPECTS

Proceedings of an International Symposium on the Effects of Therapy on the Biology and Kinetics of the Surviving Tumor, Held in Vancouver, British Columbia, Canada, February 15–18, 1989

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Preface

Effects of Therapy on Biology and Kinetics of the Residual Tumor includes the pre-clinical and clinical manuscripts presented at the International Symposium on the Effects of Therapy on the Biology and Kinetics of the Surviving Tumor, in the spring of 1989 in Vancouver, British Columbia, Canada. An attempt was made to focus the symposium on (1) the curative potential of a given therapy, (2) the behaviour of the residual tumor surviving such treatment, and (3) the short-term and long-term consequences of a given treatment as it affects the host.

There are indications that the therapy spinoffs, most of them insufficiently known and virtually unresearched, vary greatly not only in a given tumor site but also among individuals with the same tumor stage and other similar disease characteristics. At one extreme, there are situations where therapy may not lead to any documented adverse effects and where the direct tumor cell kill leading to a measurable response or cure greatly overshadows the possible hidden side effects on the surviving tumor population. At the other extreme, however, there may be situations where growth advantage of the residual tumor population after therapeutic cytoreduction does occur and could be documented if monitored properly. In those instances, the overall long-term effects of therapy may be of little benefit or may even adversely affect the host, as compared to untreated situations.

Part A of the symposium proceedings, *Pre-Clinical Aspects*, reviews in detail kinetics; kinetics and treatment design; the role of growth factors; genetic aspects of tumor growth; immunity and metastases; and the role of the microenvironment, differentiation, and chemoprevention.

Part B, *Clinical Aspects*, reviews biological response modifiers; radiobiology; clinical aspects of therapy, including issues on dose intensity, timing, and intraperitoneal administration of chemotherapy; the role of autologous bone marrow transplantation; and the short-term and long-term side effects of therapy. This latter section includes such topics as induction of second malignancies, long-term effects of radiation of breast cancer, and the quality of life.

The editors were privileged to welcome a large number of the world's leading authorities to review the key questions on tumor biology and therapy. It is the hope of the organizers that the symposium and these volumes will not only raise questions and issues of fundamental importance on tumor growth and biology, but might also serve as a springboard for further research in this area.

The organizers would like to acknowledge the assistance of Donna Watts, Lou Cox, and Betty Fata, from Venue West, Vancouver, for their assistance in organizing the convention; the below mentioned sponsors and exhibitors for their grant support to the symposium; Adria Laboratories U.S. and Ciba-Geigy Canada for their assistance in the preparation of this book; David Noble, the CCABC librarian, for his assistance in the editing of manuscripts; Mary Naylor and Fiona Buss, for their secretarial assistance; and all the symposium speakers and delegates for their stimulating presentations and discussions.

Joseph Ragaz
Linda Simpson-Herren
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QUESTIONS RELATED TO THE EFFECTS OF THERAPY ON BIOLOGY AND KINETICS OF THE SURVIVING TUMOR

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INTRODUCTION

Questions and suggestions in this Appendix were formulated by the scientific committee of the International Symposium on Effects of therapy on Biology and Kinetics of the Surviving Tumor, and reflect the input of multiple workers, invited speakers and delegates. They applied to individual sessions of the Symposium agenda, and at the time of the Convention, it was suggested that they be addressed formally, either by speakers or delegates, in an attempt to formulate a consensus on main issues raised by the Convention. These conclusions could then be offered to the medical community, both practitioners and researchers alike. It is understood that for most questions no clear answers are available, although research is intense.

It is the hope of the organizers, that the convention, and subsequently the present Volume, will assist not only with formulating identifiable recommendations for optimization of cancer therapy, but will also stimulate research in all related areas. The compiled questions reflect the key issues related to the biology and kinetics of the therapeutic intervention (figure 1).

KINETICS AND TREATMENT PLANNING:

1. Does non-curative cytoreduction in animal tumors consistently affect residual tumor cell kinetics and adversely affect the system?

2. Do phenomena accompanying non-curative cytoreduction after surgery also apply to chemotherapy and radiotherapy?

3. Outside multiple anecdotal observations, is there any scientifically substantiated evidence to support that animal data on kinetics and cytoreduction apply to human tumors?

4. If yes, should the position of incisional biopsies and other incomplete surgeries be re-examined?

5. Considering the high proportion of incomplete surgeries (particularly at diagnosis), should not there be emphasis for more research in this area?

6. Should there be attempts to increase the awareness of practitioners on these phenomena even in the absence of hard data from human studies?

7. What is the role of pre-operative and dose-intensive treatments in the context of these kinetic phenomena?

GROWTH FACTORS

1. Is there any evidence that presently known growth factors mediate growth fluctuations in human malignancies?

2. Is there any information on the role of growth factors in premalignant conditions?

3. What is the present evidence for the therapeutic potential of growth factors?

GENETIC CONTROL OF TUMOR GROWTH

What is the most recent consensus on association of oncogenic amplification and:

1. Higher incidence of certain tumors.
2. Increased risk of established cancer for more aggressive behavior.
3. Increased risk of pre-malignant condition for malignant transformation.

IMMUNITY AND BIOLOGICAL RESPONSE MODIFIERS

1. Is there any evidence implicating reduction of immunity (macrophages, T-cells, RES-system, cytokines, interferons, etc) in the mechanism for initial malignant transformation?
2. Is there any evidence that the reduction immunity will affect the rate of the dissemination and progression of established cancer?
3. What is the consensus on present data of immunotherapy, and of biological response modifiers in:
    a) restoring immunity
    b) benefits of such therapies, either alone or as adjuncts to chemotherapy or radiotherapy?
4. Are procedures such as surgery, anesthesia or generalized tissue trauma (without cytoreduction) immunosuppressive? If yes, should we expect any adverse effect on established human malignancies?

RADIOBIOLOGY

1. Is there any evidence for improved therapeutic ratio of radiotherapy if timed according to tumor kinetics (in the postoperative time period, after chemotherapy, etc)?

2. What are the most recent clinically relevant data on the role of radiosensitizers, and on the combined effect with chemotherapy?

3. What are the most recent data on improved therapeutic ratio by manipulating the tumor flow and oxygenation of radiotherapy treatments?

DIFFERENTIATION

1. Is there any evidence for conventional chemotherapy and/or radiotherapy inducing differentiation?

2. Can therapeutic agents induce in the same tumors and/or patients (either simultaneously or sequentially) both differentiation (reduction of the metastatic potential) and a mutation towards aggressivity?

3. Is there any evidence for a benefit of differentiating agents in a clinical setting?

4. What are the early results of chemoprevention in premalignant conditions?

CLINICAL ASPECTS OF THERAPY

DOSE INTENSITY

1. What is the evidence for a survival benefit of dose intensive chemotherapy and radiotherapy treatment regimens in solid tumors?

2. Are there any data on tumor kinetics of the residual tumor surviving dose intensive therapy? Any dose response effect?

3. Is there a possibility that in noncurative situations, the survival after dose intensive chemotherapy treatments of solid tumors may be reduced compared to no treatment, or to therapies utilizing low to medium treatment intensities?

4. Alternatively, if dose response in human solid tumors is established, should we utilize, in curable settings (adjuvant, low tumor bulk, etc), more routinely high dose

intensity treatments, with or without assistance of autologous bone marrow transplantation, and/or GM-CSF?

TIMING OF THERAPY

Can pre-operative therapy exert its benefit by:

1. Reduction of overall tumor burden at the time of surgery?

2. Reduction of metastatic potential of the tumor cells at the time of surgery?

3. Are there any data on benefit or adverse effects of the pre-operative treatments?

4. Is there any credence to the suggestions that the pre-operative timing of therapy without selecting the best agents of sufficient dose intensity will fail to document superiority compared to the postoperative treatment; that dose intensive therapy delivered late will also fail to do so; however, that preoperative therapy of dose effective regimens will succeed?

5. Should surgical debulking of the residual disease following chemotherapy or radiation treatment, assist in the longterm disease control?

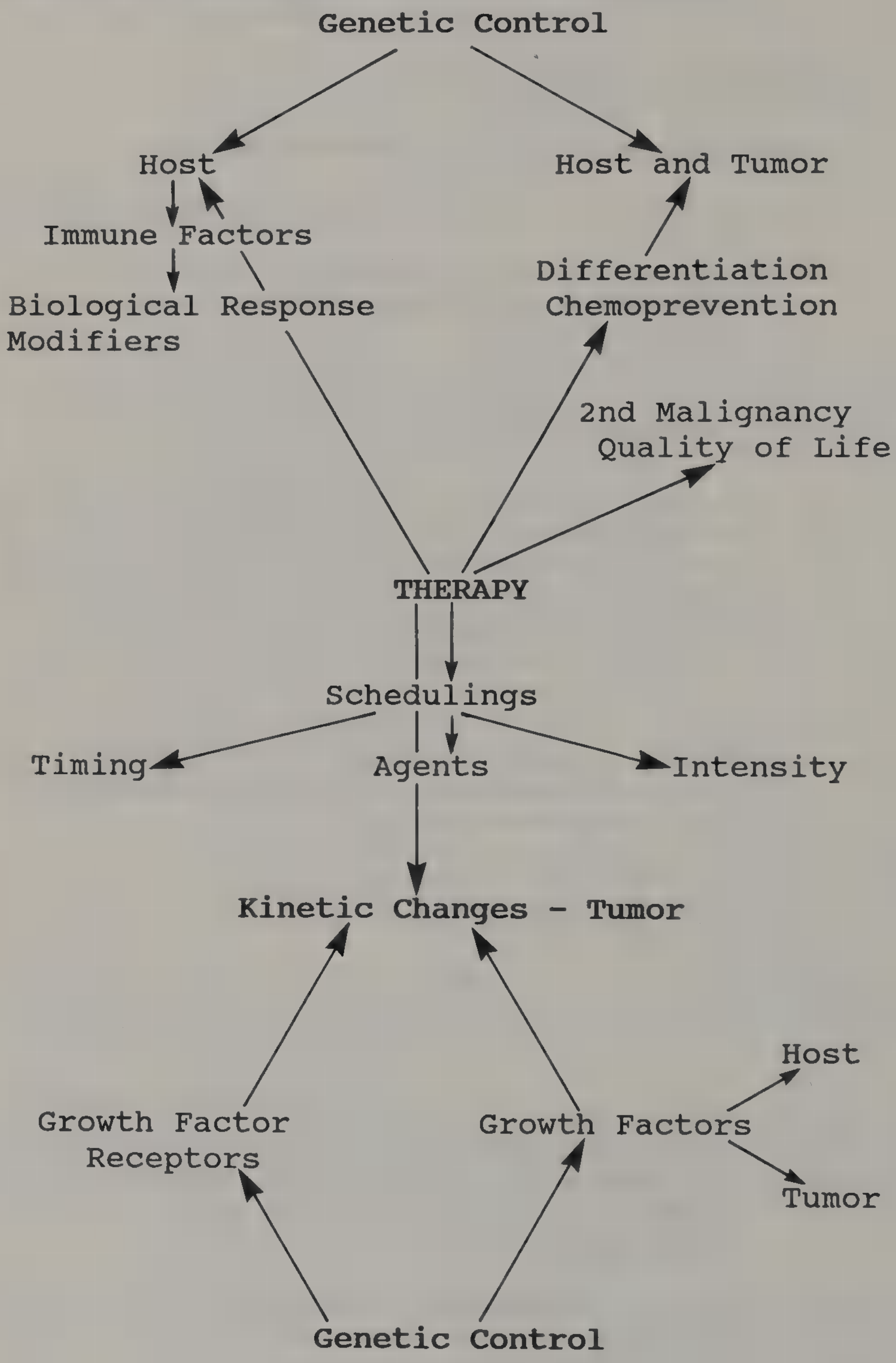
VII CONSEQUENCES OF THERAPY

1. What is the evidence that dose intensive treatments, compared to conventional therapies improve quality of life?

2. Is there any concern for a more significant emergence of second malignancies, induced as a result of utilization of high dose intensive treatment regimens in adjuvant settings, particularly targetted for large populations of young patients?

3. Are we well aware of real cost/benefit of cancer therapies?

Figure 1. **SYMPOSIUM THEME - SCHEMA**



Effects of Therapy on Biology and Kinetics
of the Residual Tumor, Part A:
Pre-Clinical Aspects, pages 7–19
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REVIEW OF THE EFFECTS OF THERAPY ON THE BIOLOGY AND KINETICS OF THE SURVIVING TUMOR. PART I.

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INTRODUCTION

The main purpose of the two volumes on Effects of Therapy on Biology and Kinetics of the Surviving Tumor is to bring into perspective the overall global effects of cancer therapy. In view of the presented data, it can be concluded that neither the tumor nor the host are passive bystanders in this process. They both contribute to the final results of a given therapy (enhancement of cell kill by host immunity, tumor and host's production of growth factors affecting tumor kinetics, etc.). The tumor and host also experience positive and adverse effects of a given treatment. An undertaking to describe all aspects of therapy, in order to be comprehensive and thorough, must include not only information on response and survival rates, but also information on other key issues of therapy, such as the mechanism of therapeutic action, as well as short-term and long-term side effects of therapy.

These, in addition to well known treatment effects such as allopecia, hematological and G.I. toxicity, must also include several of the biological therapy spinoffs such as kinetic alterations after cytoreduction, suppression of immunity, and induction of second malignancy. A thorough review of these issues indicates, that for each magnitude of a measurable therapeutic benefit, there is inevitably a concealed, but in many cases a significant behavioral alteration of the remaining tumor, with the additional accompanying side effects to the host running in parallel with the benefits of a given treatment. In most instances, it is the net balance among all the factors involved which determines the absolute therapeutic gain and overall therapeutic ratio.

Some of these aspects of therapy, most of them virtually unresearched, encouraged the editors to stage the International Symposium on the Effects of Therapy on the Biology and Kinetics of the Surviving Tumor, held in Vancouver, B.C., Canada, in the Spring of 1989, in order to establish whether the understanding and recognition of all the aspects of therapy could assist in devising more effective treatment strategies. In this first volume of the two volume Symposium Proceedings, issues such as kinetics and treatment design, growth factors, genetic regulation of tumor growth, questions on immunity, metastases, effects of the microenvironment, differentiation and chemoprevention, are reviewed in detail. Thischapter provides highlights and a brief summary of the chapters of the first volume.

KINETICS

***EFFECTS OF THE INITIAL CHEMOTHERAPY ON SUBSEQUENT THERAPY (L. Simpson-Herren).** The main emphasis of the kinetics section is its review of the effects of non-curative cytoreduction on the residual tumor, and the interaction of cytoreductive intervention with subsequent treatment planning. Simpson-Herren, one of the first to originally examine the issue of an increase of growth fraction after surgical cytoreduction, discusses here in more detail the interaction of tumor kinetics and metabolism of some chemotherapeutic agents, such as L-Pam and Adriamycin. She indicates, that biochemical resistance alone may not be primarily responsible for the variable responses to chemotherapy, and that the unequal drug distribution could be an additional major factor. Her experiments indicate that the variability of plasma level of chemotherapeutic agents is related to the instantaneous tumor burden after a given therapy, and only minimal variations of the drug levels are seen in tumor free animals. Reduced plasma levels of Melphalan and Adriamycin were seen in tumors achieving regression after that course, compared to tumors without response. Hence, measurement of chemotherapy plasma levels after its administration may be yet another indicator of response.

***PROLIFERATIVE AND VASCULAR RESPONSES TO CYTOREDUCTION IN SOLID TUMOR MODELS (P.G. Braunschweiger).** Braunschweiger describes here observations during the "proliferative recovery" after surgical or chemotherapeutic cytoreduction. He provides evidence for a suggestion that in addition to alterated kinetics, important vascular changes are also taking place in the tumor soon after therapeutic intervention. Accordingly, it could be expected, that altered responsiveness to many chemotherapeutic agents in the postoperative period may represent not only changes due to increased growth fraction after the initial cytoreduction, but also an increase of tumor vascular perfusion postoperatively, securing delivery of varied concentrations of chemotherapy. The rather profound vascular responses in the postoperative recovery time period can be monitored by techniques such as PET scan or NMR spectroscopy. It seems reasonable to expect that the recognition of these changes may offer a potential for therapeutic manipulation in the postoperative time period, such as alternating of the timing or dosing of chemotherapy and/or radiotherapy.

***REGULATION OF GROWTH IN NORMAL AND NEOPLASTIC CELL POPULATIONS BY A TISSUE SIZER MECHANISM: THERAPEUTIC IMPLICATIONS (I.L. Cameron).** Cameron et al. emphasize the importance of the tumor and tissue volume exhibiting negative feedback on the growth of its own cells, a phenomenon occurring both in tumors as well as in non-malignant tissues. Recognition of these regulatory growth mechanisms will be essential in our understanding of tumor biology, and may explain the alteration of tumor kinetics during the tumor growth and after cytoreduction. Mechanism of such negative feedback, as discussed by Fisher, Lippman and other subsequent speakers, may include an active interaction of the tumor with growth factors, elaborated either by the tumor itself, or by the host.

***SERUM GROWTH FACTOR FOLLOWING PRIMARY TUMOR REMOVAL AND THE INHIBITION OF ITS PRODUCTION BY PREOPERATIVE THERAPY (B. Fisher).** Fisher and his colleagues reported some of the original observations on increase of the growth fraction after surgery (Gunduz et al., Cancer Research, 1979; Fisher et al., Cancer Research, 1983). In their present work, not only do they reproduce their original data on the increase of growth fraction after surgical cytoreduction, but they also show that the transfer of sera from animals with surgery stimulates significantly the growth rate of animals without cytoreduction. Cyclophosphamide and several hormones given preoperatively prevent the enhancement of kinetics, bringing into perspective the value of preoperative (neoadjuvant) therapy.

This work, as well as clinical pilot projects of preoperative therapy in breast cancer by other groups have laid the foundation for the clinical trial of NSABP B18, testing in newly diagnosed breast cancer patients 4 cycles of Adriamycin - Cyclophosphamide chemotherapy given preoperatively versus the same chemotherapy started after surgery. Also, the study strongly emphasizes fine needle aspiration as the only diagnostic procedure for newly diagnosed breast cancer. This procedure, from the point of view of logistics, kinetics and safety, may be a more appropriate diagnostic procedure than the presently performed incisional open biopsy - a classical example of a noncurative cytoreduction. In addition to its safety and speed, fine needle aspiration can also provide enough material for the determination of estrogen receptors and flow cytometry.

KINETICS AND TREATMENT DESIGN

This section was designed to integrate the kinetic observations after therapy, as derived primarily from experimental data, with therapeutic planning and design of clinical studies.

***TUMOR DIFFERENCES, DRUG DIFFERENCES, TREATMENT DESIGN DIFFERENCES, AND EFFECTS ON THERAPEUTIC OUTCOME (H.E. Skipper).** Skipper calls attention to the importance of the total dose delivery as well as the dose intensity, as essential parts of all chemotherapeutic and radiotherapeutic treatment planning. In his chapter, he summarizes the most important conceptual points governing chemotherapeutic treatments in experimental systems, as previously reported by him and his colleagues.

In this respect, his chapter represents an important example of collaboration among theoreticians, and experimentalists and clinicians.

***GENETIC AND KINETIC FACTORS IN THE DRUG RESISTANCE OF MICROMETASTATIC DISEASE (J. Goldie).** Goldie discusses the genetic and kinetic aspects of treatment resistance, calling our attention to the phenomenon of tumor stem cell mutation, presumed to play an essential role in the development of therapeutic resistance. The existence of genetic markers of resistance such as the multiresistance MDR gene attests to the importance of genetic phenomena governing resistance. Identification of genes associated with resistance could very soon play an essential role in a clinical setting, permitting at diagnosis the determination of resistance. Such information may lead subsequently to specific treatment planning in individual patients. Also, manipulation of gene resistance using techniques such as monoclonal antibodies, may be utilized therapeutically - for instance in altering an established resistance, or preventing its development. Also emphasized is the significance of kinetic cycles, such as dividing vs non-dividing states, and their implication in the formation and overcoming of resistance.

***BIOLOGY OF RESIDUAL BREAST CANCER AFTER THERAPY: A KINETIC INTERPRETATION (L. Norton).** Norton discusses the mathematical modelling integrating theoretical principles of cancer therapy with clinical data seen in present and previous clinical trials. He emphasizes the need for an outline of theoretical and mathematical concepts in planning therapeutic strategies, and hence an intense interaction between theoreticians and clinicians. He offers comments regarding kinetics and treatment planning, discussing in more detail issues such as the correlation of fractional cell kill, initial tumor burden and resistance with curability. He reviews data on resistance, raising questions on when the onset of resistance starts, the applicability of the Goldie - Coldman somatic mutation theory, etc.

For most cancers, including breast cancer, he emphasizes, that parameters such as timing and dose intensity must be satisfied in a given regimen. For cross-over therapy to have impact, the selection of appropriate drugs, doses and timing must all be employed. He also discusses the possibility of the interaction of chemotherapy with host biology, such as growth factors, raising the question, for instance, whether a part of chemotherapy effect could be a suppression of stimulatory growth factors. Lastly, he stresses the importance of theoretical principles such as the application of mathematical extrapolation and curve fitting methods to the biology and the therapeutic scheduling of human tumors.

***SURGERY, KINETICS AND BIOLOGICAL CONSIDERATIONS IN PLANNING ADJUVANT THERAPY PROTOCOLS (J.F. Forbes).** In the initial part of his paper Forbes reviews the data indicating alteration of tumor kinetics after surgery. Immunosuppression, physiological stress and altered balance of growth factors are likely the principle phenomena responsible for these changes. Immunosuppression due to surgery represents in all likelihood an important biological effect, the extent of which is not yet fully appreciated. It is likely to be present in the clinical setting and is unlikely advantageous to the host. Modern detection technologies, such as those assessing the T or B cell lymphocytes, phagocytosis, or lymphocyte surface receptors, may detect immunosuppressive effects of surgery in individualized patients, whereby "tailor made" rescue therapies to restore immunity may be possible to implement.

Despite its hidden side effects, locoregional surgery remains an essential part of the initial cancer treatment, as inadequate local treatment may compromise the outcome. Tumor debulking may be an essential factor in determining locoregional or even systemic control, and both may be adversely affected by an absence of adequate locoregional therapy. In the last portion of his paper, Forbes reviews data from Ludwig studies based on theoretical observations of tumor kinetics. Ludwig's V study of perioperative chemotherapy is reviewed. While the results of peri vs postoperative chemotherapy from this trial have not confirmed the results of a superiority of early timing of chemotherapy, as seen in animal models, they nevertheless indicate the importance of treatment timing, particularly in node-negative low risk patients.

GROWTH FACTORS

***GROWTH CONTROL OF NORMAL AND MALIGNANT BREAST EPITHELIUM (M. Lippman).** Lippman summarizes the data on growth factors, updating in detail the data on growth control of normal and malignant epithelia. He brings to our attention the overall interaction between hormone actions and growth factors, implicating the mediating effect of some growth factors, either in a paracrine, or alternatively, in an autocrine fashion, for the hormonal effect. It is suspected that growth factors may play a dominant role in the initiation of malignant transformation, and also that growth factors may mediate tumor growth fluctuations of established tumor. It remains to be shown whether these mechanisms will also mediate kinetic alteration after therapeutic perturbation. Lastly, some details of biochemical and tissue interaction of growth factors and hormones, as observed "in" in-vitro experiments, are also described.

***STUDIES OF EGF - MEDIATED GROWTH CONTROL AND SIGNAL TRANSDUCTION USING THE MDA-MB-468 HUMAN BREAST CANCER CELL LINE (R.N. Buick).** Individual growth factors are discussed in the subsequent chapters of this volume. Buick presents elegant data documenting the significance of the Epidermal Growth Factor (EGF), and its interaction with other genes and receptors regulating tumor growth. EGF receptor positive and ER negative breast cancers, according to subsequent clinical data, have a distinct growth advantage, implicating EGF and its receptor in the behaviour of several human malignancies, including breast cancer. Buick also alludes to the possibility of modulating EGF and its receptor activity with monoclonal antibody. Thus, manipulation of growth factors in the clinical setting may become a reality, surpassing in its refinement, and probable absence of side effects, more crude therapeutic modalities, such as chemotherapy or radiotherapy.

***INHIBITORS OF BOMBESIN-STIMULATED INTRACELLULAR SIGNALS: INTERRUPTION OF AN AUTOCRINEPATHWAY AS A THERAPEUTIC STRATEGY IN SMALL CELL LUNG CARCINOMA (E.A. Sausville).** Sausville discusses Bombesin, another new growth factor closely linked to neuroendocrine production in tissues such as small cell lung carcinoma. Its stimulatory effects in tissue cultures, similarly to Insulin Growth Factors, render Bombesin a potent tissue and tumor mitogen. In his chapter, Sausville provides details of the biochemical interaction of Bombesin with other growth factors and oncogenes.

Other growth factors, mainly Transforming Growth Factor alpha, (TGF-alpha) and its interaction with the inhibitory transforming growth factor Beta, Platelet-Derived Growth Factors, Insulin Growth Factors, etc., are considered of great importance. In summary, it can be stated that the origin of growth factors in both nonmalignant as well as in malignant tissues, and their recovery in the blood and circulation, implicates the host regulatory mechanisms on tumor growth, and in particular, their systemic effect in the overall regulation of the total tumor burden. In view of these important data, it is easier to understand the sensitive interaction of the locoregional with the distant tumor foci, as well as why the tumor cytoreduction and other system perturbations may significantly affect the behaviour of the residual tumor.

GENETIC CONTROL OF TUMOR GROWTH

There is growing evidence that oncogenes play a significant role in tumor etiology. While their role in the initial tumor transformation has not been fully confirmed, their association with subsequent phenotypic characteristics is now more evident.

***HER-2/NEW ONCOGENE AMPLIFICATION AND EXPRESSION IN BREAST AND OVARIAN CANCERS (M.F. Press).** Press et al offer a summary of what is known on the role of HER/NEU oncogene in breast and ovarian cancer. Tumors with amplification of the gene copy had a higher expression of the gene product, accompanied by overexpression of the RNA and protein cascade. These findings allude to the possibility of a functional significance of the amplified oncogene. Indeed, preliminary evidence indicates an association of tumors with amplified HER/NEU oncogene with high risk breast cancer features and with reduced disease free survival. In the follow-up of the first study, originally described by Slamon in 1981, HER/NEU oncogene was shown in a multivariate analysis of 345 node positive breast cancer patients, to be the second most important prognostic factor after the axillary lymph nodes, to predict for disease free ($p=0.006$), overall survival ($p=0.045$). The difference in the determination of immunohistochemical assays on fresh frozen vs paraffin embedded breast cancer tissues, as well as the significance of HER/NEU oncogene, using DNA, RNA or protein assays in ovarian cancer, are also described.

***GROWTH CONTROL IN LEUKEMIA (A.C Eaves).** Recent progress in growth control of leukemia is discussed by Eaves. In-vitro cultures offer a unique environment to study the behavioral growth patterns of such malignancies such as AML and CML. They permit not only therapeutic assays for sensitivity testing, but also enable implementation of new techniques such as bone marrow purging, subsequently utilized for therapy with autologous bone marrow reinfusion. Initial overexpression and a subsequent loss of the Philadelphia (Ph) chromosome in CML in-vitro grown colonies, initially described by Eaves et al in 1985, is also discussed here. The more recent data on the growth of Ph negative vs Ph positive CML clones in culture, their interaction with growth factors, and the significance of the DNA changes as expressed in cytogenetic changes, emphasize the importance of the genetic control of hematological malignancies, similar to those observed with oncogenes in other malignancies.

In summary, it is reasonable to conclude that while technical difficulties of genetic research are presently far from being resolved, it is apparent that the genetic analysis of malignant tumors and premalignant high risk lesions may become a powerful risk determining tool in the near future. Its implication for therapy, such as prevention of gene expression, or blockade of expressed protein by monoclonal antibodies, looks extremely promising.

METASTASES AND IMMUNITY

***THE BIOLOGY OF CANCER METASTASES (J.E. Price).** Behavior of metastases including their heterogeneity and interaction with the host, and the latest genetic determinants influencing their characteristics, is discussed by Price. There is now good evidence, previously described on several occasions by Fidler et al., that even the most uniformly staged tumors exhibit a large variation and heterogeneity in the behavior of their metastases. As Price indicates, the process of establishment of metastases is not random, but depends on the intrinsic properties of the stem cell genome (the seed), interacting constantly with the host (the soil). Price describes in more detail this interaction, made more complex by the ongoing mutations of tumor cells. She concludes that new clinical protocols developed for cancer therapy will have to be based on the biological characteristics of metastases, as well as other aspects of tumor behavior.

***MACROPHAGES, METASTASIS AND IMMUNITY (N.C. Phillips).** Phillips disscusses the role of macrophages and immunity in modulating metastatic behaviour. In regards to tumor etiology, there is now good experimental evidence indicating that, while defect of macrophage function has no effect on tumor induction, the stimulation of macrophages with immunomodulators and biological response modifiers may be of great importance. A thorough review of experiments using induction of macrophage tumoricidal activity with immunomodulators such as liposomal muramyl dipeptides (LMD), is provided. Experimental evidence indicates that LMD stimulated macrophages can inhibit, for instance, murine pulmonary metastases. Furthermore, induction of hepatic Kupfer cell activity resulted in a significant inhibition of the liver carcinoma. Increased production of Interleukin-1 and of GM-CSF by LMD stimulated macrophages was also documented, indicating a possibility that one group of biological response modifiers may stimulate another group of immunomodulators by activated macrophages. In addition to a direct therapeutic application of liposomes in stimulating macrophages against developed tumors, Phillips also outlines their potential use in the prevention of tumor formation through a liposome-tumor associated antigen (TAA) containing vaccine.

***DEVELOPMENT OF MYELORESTORATIVE AND IMMUNOTHERAPEUTIC PROPERTIES OF CYTOKINES (J.E. Talmadge).** Talmadge offers an introduction to the myelorestorative and immunotherapeutic significance of cytokines. Cytokines, a term encompassing the large array of immunologically active substances produced by the host, range from clinically well tested materials such as alfa and gamma interferons, to more recent immunomodulators such interleukins and growth factors. Talmage provides a review of the initial preclinical and clinical trials of cytokines. His chapter serves as an excellent introduction to the therapeutic significance of biological response modifiers, discussed further in the second volume.

MICROENVIRONMENT, DIFFERENTIATION AND CHEMOPREVENTION

The last portion of this volume reviews the topic of the differentiation and the significance of the stroma on tumor behaviour.

***CELLULAR MATURATION AND ONCOGENE EXPRESSION DURING DRUG-INDUCED DIFFERENTIATION IN VITRO: A BRIEF REVIEW (J.L. Biedler).** Biedlers' manuscript offers a comprehensive review of the significance of differentiation, particularly as it relates to the therapeutic induction of differentiation in premalignant and malignant lesions. Agents known to induce differentiation include not only medications such as retinoids or other chemopreventive agents, but also well known cytotoxic agents such as anthracyclines, known primarily for their cell killing effects. Biedler offers convincing evidence that, in some experimental tumors such as neuroblastoma or leukemia, conventional chemotherapy may induce differentiation - an observation reported also in such solid tumors as breast and embryonal cell carcinoma.

***MAMMARY GLAND AS A MODEL FOR STUDIES OF GENE EXPRESSION IN NORMAL AND MALIGNANT CELLS. (M. Bissel).** There is now solid evidence indicating an active interaction between tumor and its neighboring stroma. Extracellular matrix in particular is especially active in regulating tumor growth. Bissel indicates that the extracellular matrix and some of its components, such as laminin, type IV collagen and heparan sulfate proteoglycan, play a fundamental role in the regulation of gene expression, both in physiological conditions as well as in developing tumors. Accordingly, the extracellular matrix is a functional extension of the cells and is to be considered an active regulator of cellular function. The influence of extracellular matrix on tumor cells includes such phenomena as differentiation, regulation of DNA, RNA and protein metabolism, oxygen saturation, etc. Indirectly, the matrix influences most other fundamental aspects of tumor behavior, including cell division and invasiveness.

***LEAKY TUMOR VESSELS: CONSEQUENCES FOR TUMOR STROMA GENERATION AND FOR SOLID TUMOR THERAPY (H.F Dvorak).** Further significance of stroma, particularly of the vessels at the tumor-stromal junction, is discussed by Dvorak. There is evidence for existence in that location of "leaky" vessels, with increased hyperpermeability to macromolecular substances, with potentially a major significance to tumor perfusion and delivery of chemotherapy. The leaky vessels are newly formed, well differentiated venules or capillaries, which respond possibly to a specific growth factor. In this respect, they differ from ordinary vessels which penetrate directly through the tumor. It is not presently known whether the leaky vessels have any association with the previously described angiogenesis factor of Folkman, or whether they are a part of inherent tumor growth. Dvorak clearly indicates that there may be major therapeutic implications as a result of these observations. For instance, small molecular cytotoxics may achieve higher tumor concentration than the large macromolecular complexes, and other similar physico-chemical drug characteristics may play a role in determining a given drug's efficacy.

***EMBRYONIC MECHANISMS FOR ABROGATING THE MALIGNANCY OF CANCER CELLS (R.E. Parchment).** Parchment's chapter includes the intriguing observations on differentiation induction by embryonal tissue. The similarity of tumor and embryo growth are striking. The embryo, but not the tumor, does have the ability, in a finite time of its history, to switch from the state of pure proliferation and invasiveness (placenta), into a state of differentiation. In the described experiments, embryonal malignant cells were injected into the active blastocyst. A conversion into nonmalignant tissue expressing the same somatic and genetic markers as the originally injected malignant tissue, was documented. Soluble factors in the blastocele fluid, considered to mediate the differentiation, were identified. In this process, the extracellular matrix is required to interact with its components such as laminin and fibronectin. Furthermore, inhibition of the growth of cells injected into the blastocele was documented, and polyamines in the blastocele were identified as main mediators with both cytotoxic and inhibitory properties. The analogy between the behavior of embryonic and tumor growth is further described by the phenomenon of "apoptosis", or programmed cell death. It indicates, that within the embryo, and in an analogy, within a tumor population, the overall control of the tumor's final volume is constantly preserved, a phenomenon largely responsible for the final balance of cell death and cell birth. Such a regulation, upon identification of soluble factors mediating this process, could be conceptually used for therapeutic purposes.

CHEMOPREVENTION

No other development pays greater tribute to the concept of differentiation than chemoprevention, a term describing the clinical utilization of naturally occurring substances for the purpose of induction of differentiation and prevention of malignant transformation.

***THE CHEMOPREVENTION OF HUMAN CANCER: AN OVERVIEW (J.S. Bertram).** Bertram's review paper on chemoprevention provides an excellent overview of the subject. Initially, he defines individual steps in malignant transformation, namely initiation, replication, promotion and subsequent progression. These are well described individual time phases, with identifiable epidemiological observations offering multiple interventional possibilities. For instance, flavones, identified in most vegetable products, can effectively alter products of chemical carcinogenesis. Ascorbic acid, by a documented blockade of nitrosamine formation, has a mode of action supporting the biochemical rationale and largely circumstantial evidence for its chemopreventive and anticarcinogenic effect.

Agents best known to act at the promotion phase include retinoids and their derivatives. According to Bertram, the protective effects of carotenoids, the largest naturally occurring supply of retinoids, have been already shown by epidemiological studies to have beneficial effect on the incidence of carcinoma of the lung, oral cavity, esophagus, stomach and bladder. Furthermore, recent animal studies have shown beneficial effects of retinoids in breast and skin carcinoma, as well as in neuroblastoma. Bertram provides a list of ongoing chemopreventive trials, indicating that in the near future, the therapeutic impact of chemopreventive intervention will be more clear.

***RETINOIDS AND BREAST CANCER (P.R. Band).** Band et al discuss in more detail the use of retinoids in breast cancer. They describe at least two major indications for clinical trials of retinoids: firstly, for the prevention of malignant transformation aimed at the high risk population; and secondly, to alter the metastatic potential of a developed malignancy by induction of differentiation. For the first utilization, selection of the high risk target population is the first step in the preparation of chemopreventive trials. Band et al. provide an effective review of the main epidemiological risk factors for breast cancer, including their development of a quantitative mean population relative risk assessment. Such quantitation includes all known risk factors in a given subset of women, integrated and calculated according to known population risk data, into a single number, expressing the relative risk for that particular subgroup. Accordingly, chemopreventive treatments of different dose intensity and duration can be devised. Also, estimates for numbers in the sample population needed for a chemopreventive trial are offered. As Vitamin A induces differentiation by controlling the gene expression, different schedules may be needed during the phases of initiation and promotion. Lastly, the authors provide some suggestions for scheduling and dosing of retinoids. It is essential to distinguish between the maintenance of normal cellular differentiation requiring only regular dietary supplements of retinoids, as opposed to the treatment of more advanced stages of tumor development, such as premalignant lesions requiring pharmacological doses of A vitamins.

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EFFECTS OF THE INITIAL CHEMOTHERAPY ON SUBSEQUENT THERAPY*

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INTRODUCTION

It is well recognized that the most effective chemotherapeutic protocols produce variable responses in patients bearing tumors of similar size and histological type. This variability is usually attributed to biochemical resistance of the tumor cell population or to heterogeneity of the host metabolism and elimination of the drug. A similar variability of response occurs in murine tumors (Schabel et al., 1982) that cannot be explained by heterogeneity of the hosts or by biochemical resistance of the tumor cell population (Simpson-Herren et al., 1987, 1988).

Variable response to effective, clinically useful chemotherapeutic agents has been demonstrated in a number of murine tumor systems including the transplantable mammary adenocarcinoma 16/C used for these studies. This tumor exhibits variable responses ranging from complete regression to growth under treatment with two clinically useful agents, melphalan (L-PAM) and adriamycin (ADR). Initially we demonstrated that the degree of variability in metabolism, hydrolysis, or elimination of these agents in individual tumor-free mice or in mice bearing 16/C tumors less than 1 g could not account for the variable tumor response (Simpson-Herren et al., 1987).

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That biochemical resistance of the tumor cell population was not responsible was demonstrated by passage into new hosts of tumors that regressed or grew under treatment with L-PAM. The second-generation tumors derived from responsive and totally unresponsive donor tumors exhibited the same spectrum of response to L-PAM as previously untreated tumors. These studies indicate that biochemical resistance was not responsible for the variable response to initial therapy but did not preclude development of resistance during extended treatment (Simpson-Herren et al., 1988).

RESULTS AND DISCUSSION

Regions of high and low perfusion within tumors were delineated by administration of a dye, lissamine green, which is distributed by the vascular supply (Goldacre et al., 1959). There was a good correlation between concentrations of L-PAM and lissamine green when tumors were dissected by visual evaluation of stain. The extractable L-PAM (or ADR) was assayed by HPLC and lissamine green was assayed spectrophotometrically. The studies indicated that concentrations of L-PAM may vary by as much as 10-fold or more between regions of high and low perfusion in a single 16/C tumor. The presence of viable clonogenic cells in the regions of low perfusion were indicated by bioassay and by measurements of mitotic and thymidine indices.

The presence of clonogenic cells in tumor regions of low drug concentration suggest that unequal drug distribution within individual tumors is a major limitation of effective therapy. Subsequent studies were designed to develop means of improving drug distribution within tumors during the course of combined and multiple-dose therapy.

In the subcutaneous 16/C tumors, the percentage of each individual tumor that was well perfused varied from less than 10% to more than 90% (Figure 1A). Poor perfusion occurred in tumors of all sizes and was not related to host status, because the maximum differences in perfusion were occasionally observed in bilateral implants in a single host.

Four days after the first, second or third dose of L-PAM (10 mg/kg) perfusion of the majority of the residual

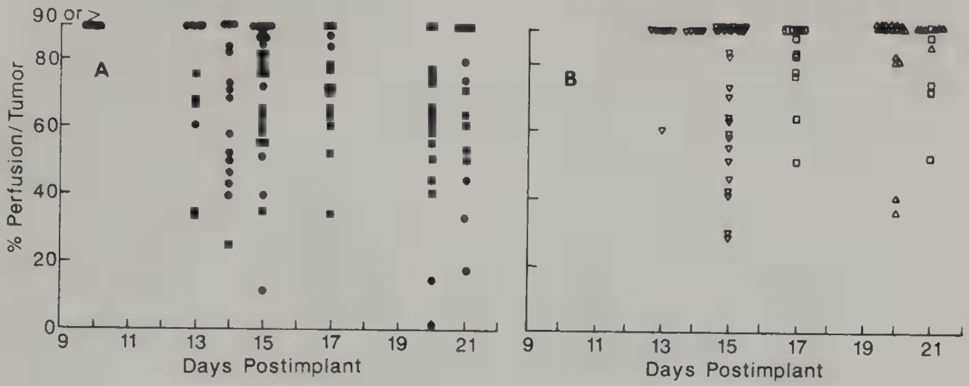

Figure 1. Relationship between age of mammary adenocarcinoma 16/C tumors and percentage of each tumor that is well perfused as delineated by lissamine green in peviously untreated mice (A) and following 1 (▽), 2 (□) or 3 (△) treatments with L-PAM (10 mg/kg) 4 days apart (B).

individual tumor nodules was improved, i.e., the fraction of individual tumors exposed to high drug concentrations was increased in the majority of tumors (Figure 1B). The improvement of perfusion during treatment may be interpreted to indicate that cells, dying due to age, lack of nutrition or lethal drug exposure, were lost from the tumor mass and the vascular supply of the residual tumor was thus improved. However, the occurrence of poorly perfused tumors at all stages of growth and during moderately effective chemotherapy suggests that the problem may be a major limitation to chemotherapy.

Plasma concentrations of L-PAM following the initial treatment of mice bearing 16/C tumors larger than 1 g were higher than plasma levels in tumor-free mice or in mice bearing tumors less than 1 g (Figure 2). The higher plasma levels of L-PAM found in mice bearing large tumors were not associated with higher levels of L-PAM in the well-perfused regions of these tumors. Plasma and tumor levels of L-PAM, in concurrent groups treated every 4 days beginning on Day 9, following treatment on Days 13 and 17 were similar. Elevation of the plasma but not the tumor level was observed on Day 21 when tumor growth during treatment was observed. Studies of the volume of distribution, drug uptake, elimination half-life, tissue distribution of ^{14}C-labeled L-PAM and its degradation products, and hematology have failed to provide a completely satisfactory explanation for these observations. Anemia develops in mice bearing large 16/C tumors but binding of drug to the

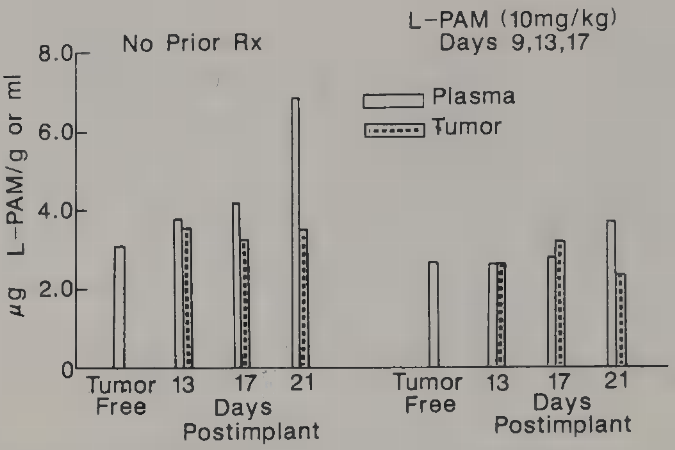

Figure 2. Plasma and tumor (well-perfused) levels of L-PAM in previously treated and previously untreated mice bearing 16/C tumors.

cellular or protein fractions does not account for the magnitude of increase in plasma levels and there was no correlation between tumor size and hematocrit.

Non-curative surgical reduction of 0.5 g subcutaneous 16/C tumors increased the time for residual tumors to reach a given tumor size but did not modify the relationship between tumor size and plasma levels of L-PAM.

Preliminary studies of dose intensification suggest that a second dose of L-PAM, administered 24 h after the initial dose resulted in slightly higher plasma drug concentrations but no significant change in the tumor levels (Figure 3). There was a decline in the tumor drug levels achieved when the interval between doses was 48 or 72 h. When the interval was 96 h, the tumor levels were slightly higher and distribution was improved. When the interval between doses was 168 h, growth was evident in all tumors and drug distribution was similar to that found in previously untreated tumors. The results suggest that the 96 h interval, which was chosen more or less arbitrarily, between doses may be nearly optimal for improvement of perfusion and tumor drug levels.

ADR differs from L-PAM in at least two ways that are important to interpretation of these results. ADR is eliminated primarily by metabolism in contrast to the elimination of L-PAM by hydrolysis. L-PAM is eliminated

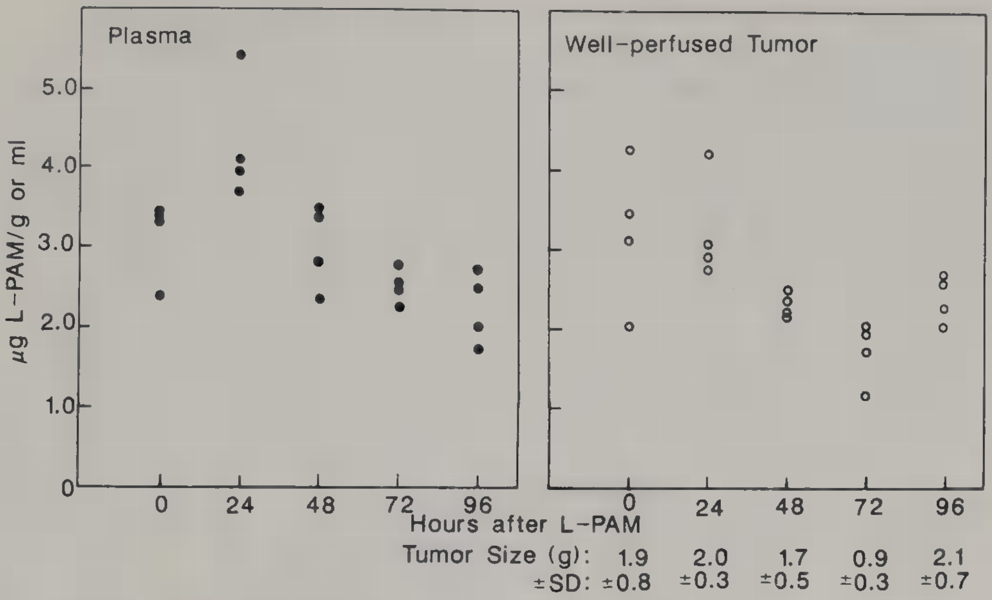

Figure 3. Effects of interval between doses of L-PAM (10 mg/kg, ip) to mice bearing 16/C tumors.

from plasma and tumor at a similar rate (Simpson-Herren, 1988) but ADR persists in tumors for extended periods.

Following ip administration of 10 mg/kg ADR, plasma levels reached an early peak and dropped almost 10-fold by 60 min (Figure 4). Tumor levels were comparable to plasma levels at 10 min and did not decrease by 60 min. Data from several studies, in which the tumor weights ranged from less than 1.0 g to more than 2.0 g were pooled. This may have contributed to the observed variability.

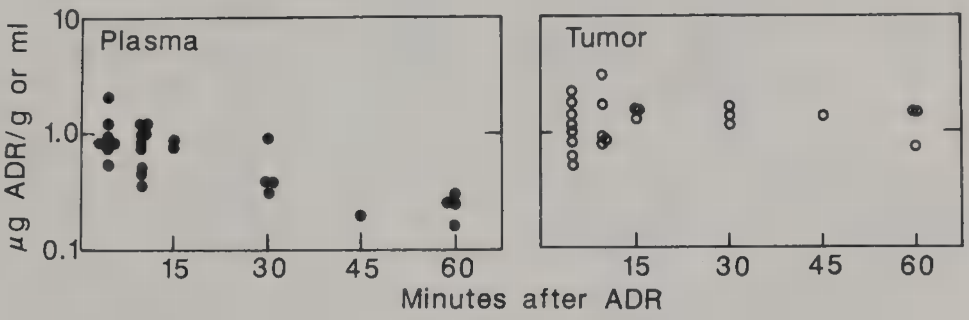

Figure 4. Following administration of ADR (10 mg/kg) ip, the tumor levels remained essentially constant in the presence of decreasing plasma levels.

Plasma levels of ADR on Day 18 postimplant in previously untreated mice bearing very large 16/C tumors were 7 to 8 times that of normal, tumor-free mice and tumor levels were less than 15 to 20% that of plasma (Figure 5). Prior treatment on Day 14 or Days 10 and 14 resulted in lower plasma levels of ADR and higher tumor levels. Whether the high tumor levels observed after the third dose of ADR resulted from improved perfusion of the residual tumor or from persistence of ADR from the earlier doses has not yet been determined. Significant build-up of drug would appear unlikely if lethally damaged cells were eliminated from the tumor mass.

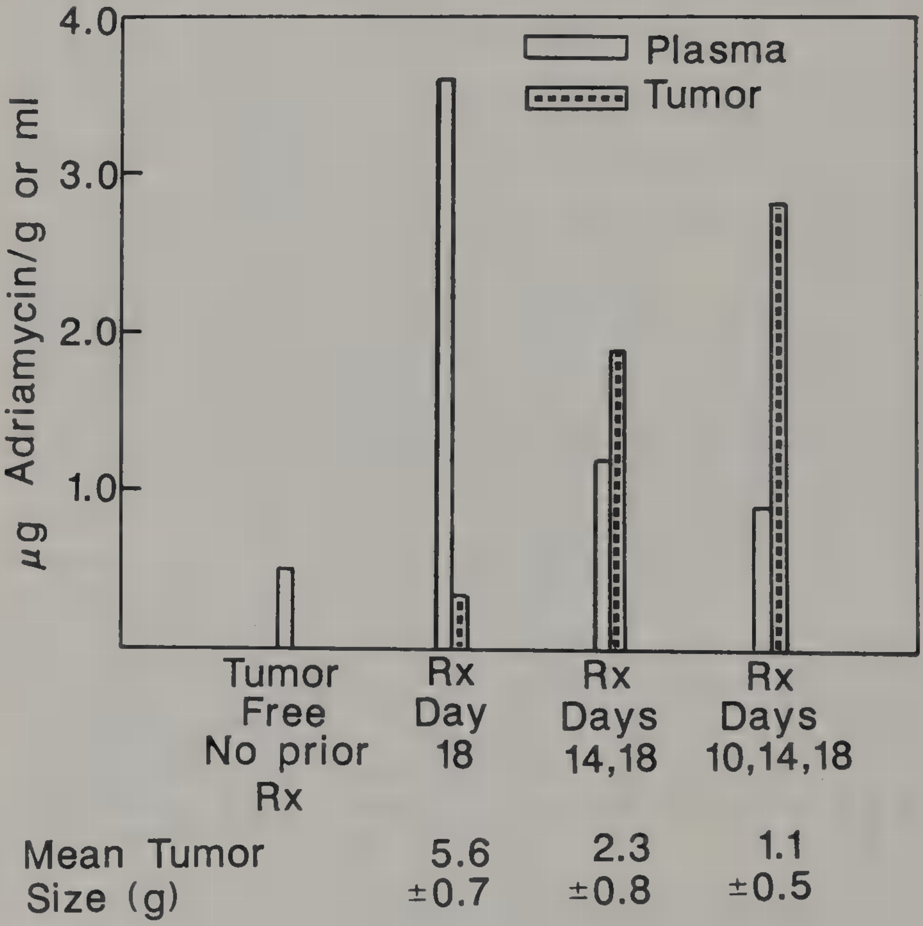

Figure 5. Levels of ADR in 16/C tumors and plasma on Day 18 following administration of ADR (10 mg/kg) ip to previously untreated and previously treated mice.

Elevated plasma levels of ADR in relation to low hematocrit values were reported for tumor-bearing rats (Broggini et al., 1980) and cancer patients (Piazza et al., 1980, 1981). This was attributed to a reduction in the amount of drug present in the cellular fraction of blood making more drug available for the plasma fraction. However, the reduction in the ADR bound to the cellular fraction of blood does not appear to be sufficient to explain the elevated plasma levels observed in our studies.

Continued variability of plasma levels during treatment may be related to the following experimental results (Figure 6). When data from multiple studies were pooled and the plasma ADR levels at 10 min were plotted against tumor weights, a relationship between tumor size and plasma level was evident. Plasma levels from mice bearing tumors

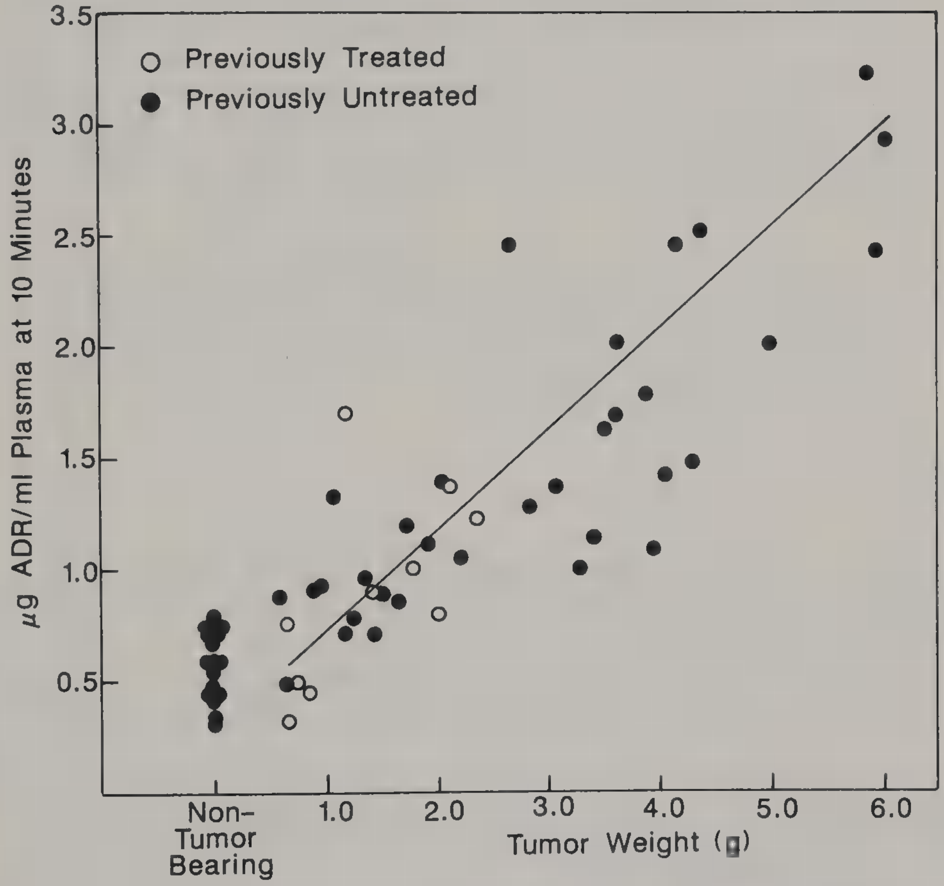

Figure 6. Concentrations of ADR in plasma at 10 min after administration of 10 mg/kg ip in relation to size of 16/C tumors. Previously treated tumors had grown or regressed as a result of earlier treatment.

that regressed or grew under previous treatment also fell within the same population of values, suggesting that plasma levels of ADR were directly related to tumor size and that tumor response may be reflected in changes in plasma levels of ADR following each dose in a multiple-dose schedule.

The studies in the 16/C mammary adenocarcinoma indicate:

1. The variability of plasma levels of L-PAM and ADR is directly associated with tumor burden. Minimal variation in plasma levels is observed in tumor-free mice and in mice bearing tumors less than 1 g.

2. The concentrations of ADR and L-PAM in advanced tumors following the initial treatment cannot be predicted from plasma concentrations.

3. The drug distribution, but not necessarily the drug concentration, is improved in the tumor surviving each treatment when the subsequent dose is administered at the appropriate time interval.

4. The plasma levels of ADR and L-PAM following each dose in a multiple-dose schedule may provide an indication of tumor response, i.e., a reduction in plasma level following equivalent doses is consistent with tumor regression.

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PROLIFERATIVE AND VASCULAR RESPONSES TO CYTOREDUCTION IN SOLID TUMOR MODELS.

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INTRODUCTION

Subcurative therapies induce marked perturbations in the proliferation kinetics of surviving tumor cell populations. Such perturbations are characterized by reduced cell proliferation, cell cycle progression delay and subsequently, by intervals of increased cell proliferation termed "Proliferative Recovery" (PR). During the PR interval, reduced cell cycle times and/or increases in the the tumor growth fraction (GF) are often noted. As most chemotherapy agents are preferentially cytotoxic for proliferating cells, the PR interval can be an interval of increased chemoresponsiveness. However, the mechanisms controlling PR are poorly understood.

Tumor cell proliferation is limited by tumor vascular patency and by tumor blood flow. Early work by Tannock (Tannock I.F., 1968) and others (Hirst, D.G. and Denekamp, J., 1979) indicated that cell proliferation and tumor growth fraction decrease with increasing distance from the tumor capillary. Recent evidence indicates that many steroid hormones and chemotherapy can affect a variety of vascular responses in solid tumors and corresponding normal target tissues. For example, in the ventral prostate of castrated rats, testosterone replacement induced increases in organ blood flow prior to expected increases in cell proliferation (Braunschweiger et. al, 1986a). Corticosteroid hormones were also shown to affect tumor vascular permeability, tissue water distribution and tumor blood flow in model systems (Braunschweiger, P.G. and Schiffer, L.M., 1986; Braunschweiger et. al., 1987). PR after chemotherapy can also be accompanied by rather profound changes in tumor vascularity, vascular permeability and tumor blood flow (Braunschweiger, P.G., 1988).

The present experiments were undertaken to further investigate dose and tumor specific PR characteristics induced by cDDP and to investigate changes in tumor blood flow attendant with PR. Using a metastatic tumor model, data is presented which supports the idea that PR can be exploited to increase the efficacy of chemotherapy.

METHODS

The RIF-1 tumor model, originally obtained from Dr. R. Kallman (Stanford University) was maintained *in vitro* and passaged *in vivo* in 8-10 week old female C3H/HeJ mice, as described previously (Braunschweiger, P.G. and Schiffer, L.M., 1983). The Lewis lung and M5076 reticular cell sarcoma cell lines were obtained from the NCI tumor bank and maintained in 8-10 week old C57Bl/6J mice by trocar implantation of tumor fragments from 14 day donor tumors. All mice were obtained from Jackson Laboratories (Bar Harbor, ME) and quarantined for two weeks prior to entering studies. All animals were housed 4 mice/cage in a temperature and humidity control animal facility with a 12 hour light-dark cycle. Randomly chosen mice were tested and found to be free of adventitious murine viruses. All mice were fed standard mouse chow (Purina, Evansville, IN) and water *ad libitum*.

Cis-diaminedichloroplatinum II (Platinol, cDDP, Bristol Laboratories) was obtained from the National Cancer Institute (DCT), diluted with sterile, non-pyrogenic water and administered by i.p. injection in a total volume of 0.2 ml. Controls received diluent alone.

Two surgical procedures were used for these studies. Partial tumor resection, approximately 75% of the tumor mass, was accomplished, as described before, under ether anesthesia (Braunschweiger, et.al, 1983). Local bleeding was controlled by cauterization. Surgical cure of intramuscular (gastrocnemus muscle) M5076 tumors was accomplished by leg amputation under ether anesthesia. The femoral artery was ligated and the tumor-bearing hind limbs were removed, *in toto*, by disarticulation of the femur from the acetabulum. Inguinal nodes and fat were also removed. Bleeding was minimal and surgical deaths were invariably anesthesia related.

3H-TdR labeling indices were determined for RIF-1, Lewis lung and M5076 tumors by an *in vitro* labeling method and autoradiography (Braunschweiger, P.G. *et al*, 1977). In some studies with the RIF-1 tumor model, proliferation in the clonogenic cell population was determined by the hydroxyurea suicide technique (Braunschweiger, P.G. *et al*, 1982). In studies to determine the effect of primary tumor amputation on the proliferation of M5076 cells in liver micro-metastases, *in vivo* 3H-TdR labeling and autoradiography was used. Mice were given 3H-TdR (0.5 uCi/g body wt; 20 Ci/mM; NEN, Boston, MA) by i.p. injection and killed one hour later. Livers were resected, weighed, fixed in phosphate buffered formalin. Paraffin sections (4 micron) were prepared and autoradiogramed. Exposure times (3-4 weeks) were determined by test sets and background was less than 1 grain per equivalent cell area. Labelling indices for M5076 cells in micrometastases were determined by counting at least 200 cells/liver from each of 5 mice/group.

Using a similar experimental design, metastatic tumor burden was quantitated by the uptake of 3H-TdR in the liver from tumor-bearing and non-tumor-bearing controls. In these experiments, 3H-TdR was injected i.p. and livers resected at one hour, as above. The livers were weighed, homogenized and an aliquoit soluabilized in tissue soluabilizer (NEN) for liquid scintillation counting. The tritium radioactivity was determined and the total liver uptake (dpm/liver) calculated. The dpm/liver in tumor-bearing mice, minus the dpm/liver in non tumor-bearing mice was taken to reflect tumor burden. Experiments were conducted between days 10 and 28 after tumor implantation and the doubling time (DT) for tumor burden determined by exponential least squares regression analysis.

The tumor Growth Fraction (GF) was estimated by the PDP assay described previously. This autoradiographic assay was shown to provide a reliable estimate of the growth fraction in tumor model (Braunschweiger, P.G., 1986).

Tumor blood flow was determined by the ^{86}RbCl distribution technique (Saperstein, L.A. 1959) as used by us previously (Braunschweiger, P.G., 1988). Since ^{86}RbCl distribution, an indirect measure of regional blood flow, is dependent on cardiac output, the uptake of ^{86}RbCl, as a percent of the injected dose (ID) per gram (dry weight) of tissue, was multiplied by the mouse body weight (Braunschweiger, P.G. et al, 1988). Tissue water content and dry weights were determined by heating to constant weight at 105° C.

Animal median survival times were determined in studies to evaluate the efficacy of surgery, cDDP chemotherapy combinations. cDDP was given one day prior to total tumor resection by leg amputation or at various intervals after surgery. Diluent + surgery controls were carried. Median survival times were determined in cDDP and control groups and the increase in median survival (IMS) determined.

Assuming that the mice in the survival experiments die as a result of metastatic tumor burden, the reduction in metastatic tumor burden necessary to produce a specific IMS can be estimated. This *in vivo* apparent surviving fraction (SF) is calculated from:

$$SF = e^{(-LN\ 2)\ (IMS/Td)}$$

where IMS is the increase in median survival time (days) and Td is the doubling time for the metastatic cell population, determined in the 3H-TdR uptake studies described above. Exponential least squares regression analysis was used to describe the cDDP dose response relationship and construct survival curves. Do's were estimated as the reciprocal of the exponential slope. A similar approach has been used previously to estimate apparent cell kill from regrowth delay measurements (Skipper and Schabel, 1984).

In time course studies, analysis of variance and the Newman-Kuels multiple range test were used to determine the significance of post-therapy changes in 3H-TdR LI and PDP indices. The test for medians and Fishers Exact test was used to determine the significance of observed increases in animal median survival. In all instances, a P value of less than 0.05 was considered adequate to reflect the null hypothesis.

RESULTS

Figure 1. shows the results from experiments with RIF-1 tumors to compare PR after 4 and 8 mg/kg cDDP. Decreased cell proliferation was seen within 2 days. In the 4mg/kg study, recovery was apparent by day 3 and on days 4 and 5 3H-TdR LI's were significantly greater than those seen in controls. In the 8 mg/kg study, the onset of PR was delayed, as control LI's were not seen until days 5-6, and peak LI's were not seen until day 7.

The proliferative response to cDDP was not only dose dependent, but also specific for the tumor model studied (Figure 2). In the Lewis lung tumors PR was initiated 3-4 days after 8 mg/kg cDDP with peak LI's seen on days 5 and 6. In the M5076 tumor model, PR after the same ip. dose was not seen until day 6.

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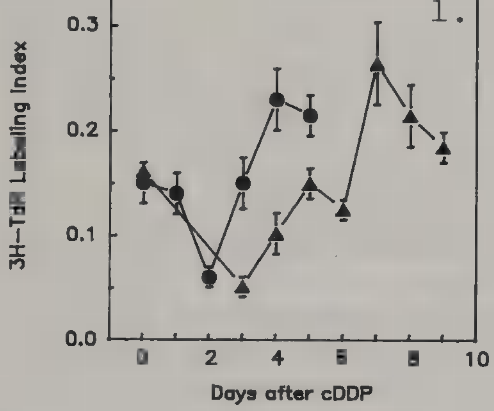

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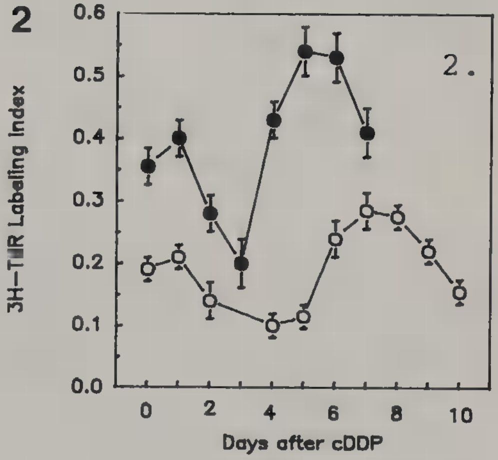

Figure 1. The effect of 4 (●) and 8 (▲) mg/kg cDDP on 3H-TdR LI for 5 RIF-1 tumors/group.

Figure 2. The effect of 8 mg/kg cDDP on the 3H-TdR LI of Lewis lung (●) and M5076 (○) tumors n=5-6 tumors.

Surgical cytoreduction can induce marked proliferative responses in residual tumor cell populations (Figure 3). Decreased cell proliferation in residual RIF-1 tumors, after partial (3/4) resection, was followed by increases in the tumor GF (days 3-5 after surgery) and the fraction of DNA synthesizing clonogenic tumor cells (days 4-5). Since previous studies indicated a relationship between PR and tumor GF (Braunschweiger, P.G., 1986), experiments were conducted to test the hypothesis that an increased GF would result in a delay in the onset of PR after cDDP. In these experiments (Figure 4), cDDP (4mg/kg) was given to mice with intact RIF-1 tumors and to mice 3 days after 3/4 tumor resection. In the surgery + cDDP group, the PR was delayed as compared to that in intact tumors. Peak LI's were seen on days 6-7, while in intact tumors, peak LI's were seen on days 4 and 5.

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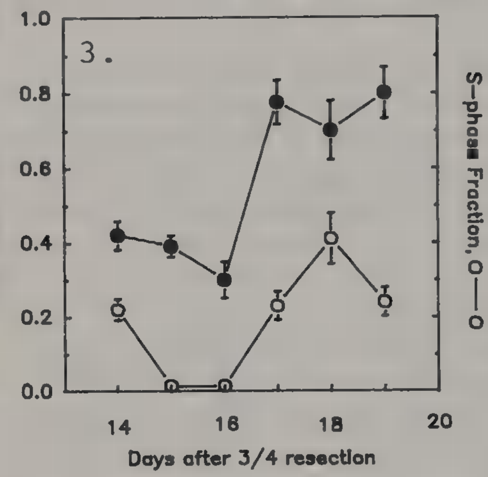

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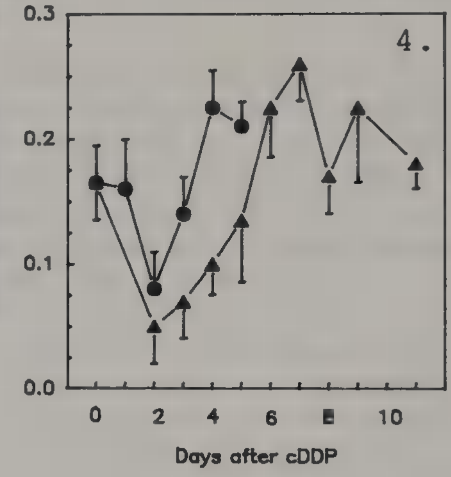

Figure 3. The effect of partial tumor resection on the PDPI (●) and S-phase clonogenic fraction in residual RIF-1 tumors; Braunschweiger, P.G. et al, 1983, with permission.

Figure 4. The effect of 4 mg/kg cDDP on the 3H-TdR LI of RID-1 tumors. ● , cDDP in intact tumors; ▲ , cDDP given 3 days after partial tumor resection. n=5-6 tumors.

Partial tumor resection also produced profound effects on the blood flow in the residual RIF-1 tissue (Figure 5a). In control tumors, perfusion decreased with increasing tumor age (i.e.,size). In the surgery group, tumor blood flow decreased within 1 hr and reached a nadir by 24 hrs after resection. Tumor blood flow was re-established by 48 hrs and by 72 hr, perfusion was significantly greater than that in age matched controls.

Thus in the RIF-1 tumor model partial tumor resection resulted in a prompted decrease in tumor blood flow and decreased tumor cell proliferation. Increases in the tumor growth fraction signaled the onset of PR in the residual tumor, but increases in tumor blood flow preceded increases in the RIF-1 tumor GF and cell proliferation. Further, these physiologic and biochemical responses to surgical cytoreduction preceded measurable tumor regrowth (Figure 5b).

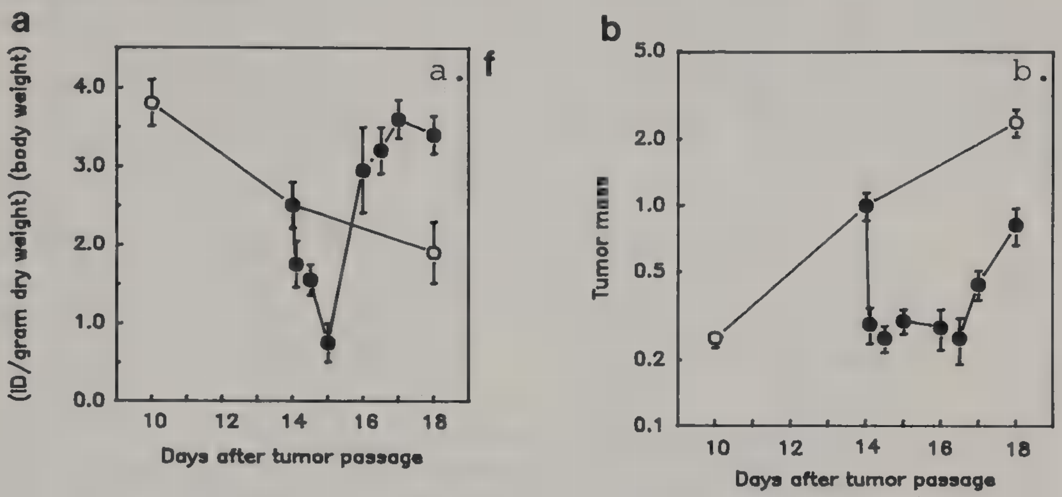

Figure 5. The effect of partial surgical excision on blood flow (5a) and growth (5b) of residual (●) and untreated control (○) RIF-1 tumors.

In previous studies (Braunschweiger,P.G., 1986), the time for PR after cyclophosphamide correlated with the tumor GF at the time of treatment. A similar relationship between PR and tumor GF is apparent for the cDDP (Figure 6). The dose (mg/kg) dependent time (dT) for PR, after cDDP treatment, is expressed as a multiple of the tumor specific cell cycle time (Tc). Regardless of whether dT is the time to peak LI or the time to reach control LI after cDDP treatment, the time to PR correlates well with the pretreatment GF.

The M5076 tumor model spontaneously metastasizes to the liver within 14 days after intramuscular implantation of tumor fragments. The effect of primary tumor amputation on cell proliferation in liver micrometastatic foci is shown in Figure 7. Decreased 3H-TdR LI's indicated siginificantly decreased cell proliferation for approximately 3 days after surgery. Increases in the 3H-TdR LI on days 4-8 suggested an interval of proliferative recovery.

Metastatic tumor growth was quantitated by 3H-TdR uptake (Figure 8). In this representative study, exponen-

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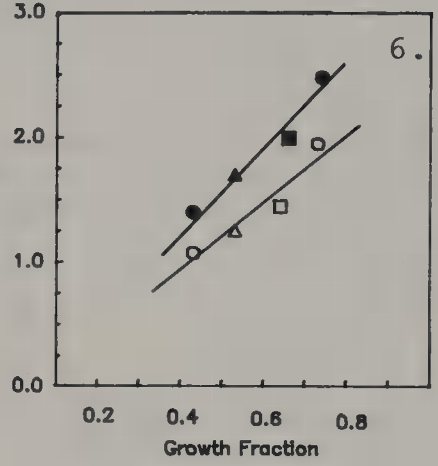

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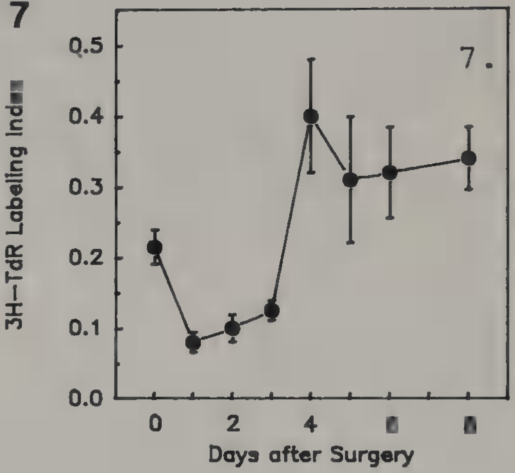

Figure 6. The correlation between the time for PR (dT/Tc/mg/kg) after cDDP and pretreatment tumor growth fraction, for RIF-1 (●,○), M5076 (▲,△) and Lewis lung (■,□) tumors. Closed symbols - dT = time to peak 3H-TdR LI; open symbols, dT = time to reach control LI.

Figure 7. Effect of the excision of primary intramuscular M5076 tumors by leg amputation on the 3H-TdR LI of M5076 tumor cells in liver micrometastases.

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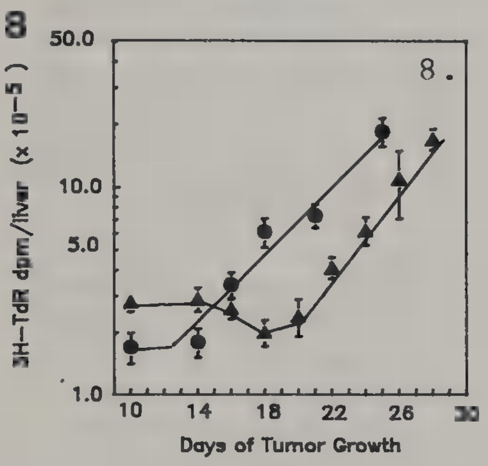
tial least squares regression analysis indicated a doubling time of 4.3 days in intact mice. After primary tumor excision, a 4 day growth lag was seen, and the doubling time was 3.8 days. Increases in tumor mass, determined by weighing livers from tumor and nontumor-bearing controls, gave similar mass doubling times. 3H-TdR uptake doubling times ranged from 3.8 to 5.2 days with a mean of 4.3± 0.4 (1 SD) days.

The results from earlier studies suggested that the PR interval of increased cell proliferation after surgery can correspond to intervals of increased chemoresponsiveness. M5076 tumor-bearing mice were treated with 4 mg/kg cDDP at various times relative to primary tumor excision (figure 9). Also shown are the results from a similar study with mice bearing a cDDP resistant M5076 line designated M5076/rP. cDDP (6mg/kg) had little or no effect on the survival of mice bearing M5076/rP tumors. On the other hand, increases in median survival were seen for mice bearing the M5076 tumor model treated with 4 mg/kg cDDP. Significantly prolonged survival times were seen for M5076 tumor-bearing mice treated with 4 mg/kg cDDP on days 3, 4 or 7 after primary tumor excision. Figure 10a shows the relationship between cDDP dose and increased median survival times for mice treated with cDDP one day before primary tumor excision or at 2,3 or 4 days after tumor excision.



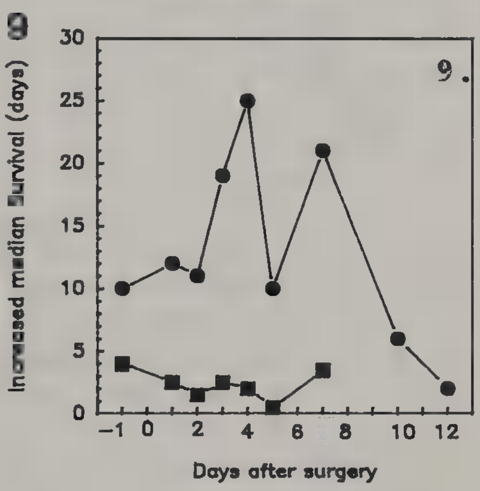


Figure 8. The effect of primary M5076 tumor excision on the growth of M5076 liver metastases. 3H-TdR dpm/liver in tumor-bearing mice - dpm/liver in age matched nontumor-bearing mice. ●, Sham surgery; ▲ , tumor amputation at day 14.

Figure 9. Increased median survival for mice bearing M5076 (●) and M5076/rP (■) tumor treated with total tumor excision (leg amputation) and cDDP, 1 day before or at several intervals after surgery. Each pt represents the difference in median survival of 10 surgery + cDDP mice and 10 surgery alone mice. Using the median test, a 5 day increase in median survival was significant at 0.05.

The average median survival times for four groups of surgery + cDDP vehicle controls was 17.3 ± 1.3 (±1SD). In the controls, cDDP 1 day prior to surgery resulted in a 2 component dose response relationship. At 2 days after surgery, the cDDP dose response was similar to that in the controls. When tumors were treated on days 3 or 4 after surgical excision of the primary tumor, a single component linear dose response relationship was seen.

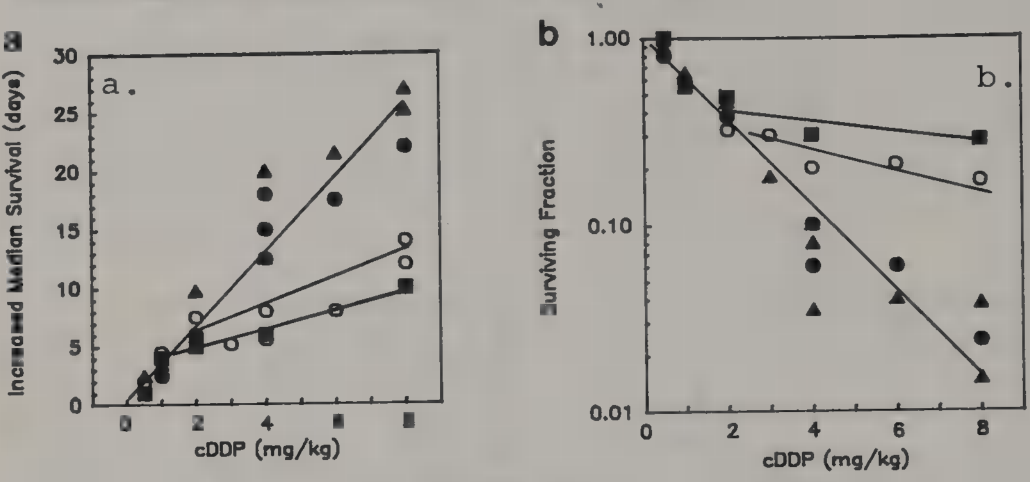


Figure 10. cDDP dose dependent changes in animal median survival times (10a) and apparent metastatic cell survival (10b) in mice treated with cDDP 1 day prior (○) or at 2 (■), 3 (▲) or 4 (●) days after tumor excision. Each pt represents the difference in survival for 10 mice treated with surgery and cDDP and 10 mice treated with surgery alone.

Assuming that tumor-bearing mice die as a consequence of metastatic tumor burden, the reduction in tumor burden to increase median survival can be estimated if the tumor doubling time is known. Using the IMS data presented in Figure 10a and the doubling times for metastatic cell foci determined in experiments similar to that described in Figure 8, apparent surviving fractions for M5076 metastatic cell populations treated with cDDP before or after primary tumor excision were calculated (Figure 10b).

When cDDP was given prior to primary tumor excision, (controls), the resulting dose response survival curve demonstrated an α (Do ~1.8mg/kg) and a β (Do ~5.6 mg/kg) component. Extrapolation of the component to the Y axis suggests that the more resistant population is approximately 45% of the total metastatic tumor cell population. In the 2 day study, cDDP sensitivity was similar to that for controls. However, a single component, exponential relationship between apparent SF and cDDP dose was seen when cDDp was given on days 3 or 4 after surgery. The Do values for cDDP in the 3 and 4 day studies were 1.9 and 2.1 mg/kg respectively.

DISCUSSION

Although proliferative recovery in solid tumors after cytoreductive therapies has been identified as an interval of increased chemoresponsiveness, little information on dose response characteristics and mechanisms controlling tumor repopulation after cytoreduction is available. The cell kinetic perturbation induced by chemotherapy (Braunschweiger, P.G., 1986), radiotherapy (Braunschweiger, P.G. _et al_, 1981), corticosteroids (Braunschweiger, P.G. _et al_, 1983) and surgical excision (Braunschweiger, P.G. and Schiffer, L.M., 1983) is characterized by a variable interval of reduced cell proliferation. In the present studies, this interval was cDDP dose dependent. After non-curative therapy, intervals of increased cell proliferation ultimately lead to the rapid tumor regrowth. This PR interval is charactized by increases in the fraction of cells synthesizing DNA and, often, by an increase in the growth fraction (Simpson-Herren, L. _et al_, 1979; Braunschweiger, _et al_, 1983; Ogita, S. _et al_, 1985). As in previous studies with cyclophosphamide, the time for PR, after cDDP, correlated with the pretreatment tumor GF.

In RIF-1 tumors, increases in the tumor blood flow were seen prior to increases in tumor GF and tumor cell proliferation during PR. Such findings might suggest that vascular responses play an important role in initiating PR and in supporting increased proliferative activity during the PR interval.

In the RIF1 tumor model, increases in tumor blood flow and vascular volumes preceded or accompanied PR after cyclophosphamide (Braunschweiger, P.G., 1988) or multiple treatments with dexamethasone (Braunschweiger, P.G. and Schiffer, L.M., 1986). Thus, increased chemoresponsiveness in residual solid tumor during PR may not only reflect a transient cell kinetic response (e.g. recruitment) but also increased drug delivery as a consequence of increased tumor blood flow.

Primary tumor resection can induce transient proliferative responses in distant metastases. In RIF1 artificial lung metastases, increases in cell proliferation were seen after the amputation of primary tumors (Braunschweiger, P.G. et al, 1983). In the present studies, 3HTdR labeling studies indicated that cell proliferation in spontaneous M5076 liver micrometastatic foci was markedly increased on days 4-8 after primary tumor amputation. Animal survival studies indicated that the best cDDP treatment intervals corresponded to post-surgical intervals of increased cell proliferation in the micrometastatic foci. Previous studies in Lewis lung tumors also indicated that the efficacy of surgery-chemotherapy combinations were highly time dependent (Braunschweiger, P.G. et al, 1984). c-DDP time and dose response studies indicated that M5076 liver metastases contained cell populations with variable c-DDP sensitivity. The data is consistent with the hypothesis that the more resistant population (Do 5.6mg/kg) was approximately 45% of the total metastatic cell population. Between days 2 and 3 after primary tumor excision, resistant cells decreased dramatically such that by days 3 and 4, the dose response indicated a more homogeneous cell population with increased cDDP sensitivity. Since it is unlikely that phenotypic resistance could be reversed so rapidly *in vivo*, and since cDDP is more toxic to actively proliferating cells than to non-proliferative cells (Roberts, J.J. and Fravel, H.N.A, 1980), the results probably indicate that between days 2 and 3 after surgery, the GF of metastatic foci increased. Whether liver blood flow is also increased is yet to be determined.

The mechanisms controlling the onset of proliferative recovery after cytoreduction are not well understood. In the RIF-1 tumor, marked increases in tumor edema (Braunschweiger, P.G., 1988) were seen after chemotherapy. During PR, edema decreases as tumor blood flow and the capillary permeability, surface area product increase (Braunschweiger, P.G., 1988). These observations lead to the interesting possibility that tumor vascular responses secondary to tumor cytoreduction provide a physiologic control of proliferative recovery.

Since corticosteroid hormones such as dexamethasone are known to have antiproliferative and vascular effects in solid tumor models (Braunschweiger, P.G. et al, 1988; Braunschweiger, P.G. and Schiffer, L.M., 1986), hormonal responses to the surgical stress associated with anesthesia and tumor excision may influence blood flow and cell proliferation in local or distant residual tumor. Previous studies, in solid tumor models indicated that the post-surgical interval of reduced cell proliferation was not only dependent on intact adrenal function, but also on the corticosteroid receptor content of the tumor model being studied (Braunschweiger, P.G. and Schiffer, L.M., 1986).

Proliferative recovery after chemotherapy is not only characteristic of tumors but also of dose limiting normal tissues. Unlike the hematopoietic cell renewal system which has a predictable recovery pattern from cytotoxic insults, the variable time course of PR in model systems would suggest that, in heterogenous patient populations, response variability may be even more profound. Such conditions make the application of cytokinetically based therapeutic strategies logistically difficult unless reliable approaches to serially monitor responses can be developed. Our findings that PR can be accompanied by rather profound vascular responses provides a rational for the application of several new technologies to serially monitor the pathophysiologic responses in treated tumors that are attendant with proliferative recovery.

Positron emision tomography (PET) is a new imaging modality which has been used to study regional blood flow and metabolic activity. Evelhoch (1986) used this method to study blood flow in RIF-1 tumors by an ^{15}O washout technique. Since ^{15}O has a short half life and can be generated _in vivo_, by photon irradiation, this technique could be applied for the serial evaluation of tumor blood flow.

In vivo 31PNMR spectroscopy is another emerging technology with great potential for non-invasive serial studies of tumor bioenergetics. Previous studies in model systems would suggest that the age dependent decreases in tumor bioenergetics is closely correlated with attendant age related decreases in tumor blood flow (Evelhoch, J.L. _et al_, 1986; Evanochlco, W.T. _et al_, 1983, 1984). Further, increases in the bioenergetic status and pH of RIF-1 tumors correlated with proliferative recovery and increases in tumor blood flow after a single cyclophosphamide treatment (Schiffer, L.M. _et al_, 1986; Li, S.J. _et al_, 1988; Braunschweiger, P.G., 1988).

1HNMR imaging techniques with and without paramagnetic contrast may also be useful for serial studies of pathophysiologic changes in solid tumors after therapy. In vivo, proton spin-lattice (T1) and spin-spin (T2) relaxation times can be greatly affected by changes in capillary permeability, water content and tissue solute distribution (Braunschweiger, P.G. and Rich, M.A., 1987). Indeed, such changes are the basis for differential 1H-NMR imaging of normal and tumor tissue (Bottomley et al, 1984). Since cytoreductive therapies have also been shown to induce rather profound changes in the distribution of tissue water and solute, tumor blood flow and capillary permeability (Braunschweiger, P.G., 1988), this readily available imaging modality may be extremely useful to serially monitor therapeutic responses (Braunschweiger, P.G. and Rich, M.A., 1987). Preliminary data would suggest that this approach may be especially useful in protocols involing hormonal manipulations (Braunschweiger, P.G. et al, 1986a, 1986b, 1986c, 1986d).

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SERUM GROWTH FACTOR FOLLOWING PRIMARY TUMOR REMOVAL AND THE INHIBITION OF ITS PRODUCTION BY PREOPERATIVE THERAPY

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Introduction

For three-quarters of a century, reports of clinical and laboratory investigation have related the presence or removal of a primary tumor to the growth of metastases.[8,15] How a primary tumor influences metastases, however, is unclear. Few investigators have determined the effect of removal or manipulation of a primary tumor on the kinetics of metastasis as measured by DNA synthesis of tumor cells. Our own investigations[2-6,9] and those of others[1,12,14] have demonstrated that, following removal of primary tumor but not after amputation of a normal leg, changes occur in the kinetics of cells in a distant tumor focus and that perturbation of a primary tumor by radiation also results in similar kinetic changes. These findings led us to conclude that accelerated tumor growth following removal of a tumor was probably the result of conversion of noncycling G_0 cells into proliferating cells.

It was felt that these findings would have greater clinical significance if (a) additional laboratory models demonstrated the kinetic changes observed following tumor removal, supporting the generality of the phenomenon, and (b) there was insight into the mechanism for the findings. It was also deemed important to determine whether the administration of local or systemic treatment prior to tumor removal (TX) could alter the cytokinetic findings. This report provides information from six host tumor systems to determine the universality of the kinetic changes observed following tumor removal. It also presents observations which indicate that the stimulation of labeling index (LI) in a distant tumor focus following tumor removal is related to the presence in the serum of a growth-stimulating factor. Finally, it indicates that the administration of local or systemic treatment prior to primary tumor removal inhibits both the production of and response to the stimulating factor, a finding with significant clinical implications. A detailed presentation of these findings appears in two recent reports.[5,6]

MATERIALS AND METHODS

Experimental Animals and Tumors Employed: The origin, supplier, and strain of mice carrying the tumors used in these experiments are presented in Table 1. Young adult females were used in all studies.

Table 1

INFORMATION REGARDING MOUSE TUMORS EMPLOYED

TUMOR	STRAIN CARRYING TUMOR (ALL FEMALES)	TUMOR ORIGIN
C3H Mammary CA	C3HeB/FeJ	Viral in C3H/HeJ Female
MXT_a MXT_b Mammary CA	$BD2F_1$	Urethane Induced in C57BL X DBAJF Female
MC54 Sarcoma	C3HeB/F3J	MC Induced in C3HeB/FeJ Female
CD8 Mammary CA	CD8F	Viral in Balb/C X DBA/8F
3LL Lewis Lung	C57BL	Spontaneous in C57 Bl Male

Preliminary experiments were done to determine the size of inoculum required to produce a 3-5 mm tumor in the left leg and a 5-7 mm tumor in the right in 14 days. In all animals containing two tumor foci the larger is designated as the "primary" and the smaller one as the "metastatic" or residual focus. Serum recipients were animals containing a single tumor focus which was similar in size and duration of growth to the smaller of the tumors in animals with two foci. Tumors were removed by amputation of the leg under light ether anesthesia.

Labeling Index (LI): The LI was determined by autoradiography employing single-cell suspension incubated with [H^3] thymidine (14 to 17 Ci per mmol). The method has been repeatedly described[5,6].

Irradiation (XRT) of Primary Tumors: Irradiation of tumors was carried out by anesthetizing mice with pentobarbital sodium. They received 50 Gy from a Linear Accelerator, Therapi 4 in a period of 12 minutes. Control animals were sham radiated, that is they were anesthetized with pentobarbital for the same period of time as were the radiated animals.

Drug Administration: Cyclophosphamide (CY) was diluted so that 240 mg/k was contained in 0.01 ml/g body weight. It was administered i.p. as a single dose 5 days prior to tumor removal or to tumor bearing recipients 5 days before their inoculation with sera. Zoladex (Z, ICI 118,630) is a luteinizing hormone-releasing hormone (LHRH) analog, that induces pituitary down regulation resulting in a medical castration which is reversible on cessation of therapy. It is administered as a sustained release depot containing 0.5 mg of the drug. The depots were implanted subcutaneously on the abdomen of the mice 14 days prior to tumor cell inoculation and were present during the entire experiment. Tamoxifen (TAM, Nolvadex®) is a nonsteroidal compound with estrogenic activity in the mouse. As the free base in powder form, it was mixed with laboratory chow at a concentration of 1 mg/g ground chow and fed to mice from the day of tumor cell inoculation and throughout the entire experiment. Both the Zoladex and the tamoxifen were supplied by ICI Pharmaceuticals, Wilmington, Delaware.

Serum: Mice were bled from the retroorbital plexus, the serum separated at once and stored at -90° until used. Sera were collected either with the tumors in place or at intervals after primary tumor removal. Serum from non-tumor mice was also collected. One ml of serum was administered to recipient mice intra-peritoneally.

In-Vitro Incubation: Each experiment was carried out employing cells from a different C3H tumor as the target. Tumors growing for approximately 14 days in untreated C3H mice were removed, minced in Eagle MEM with no serum. The cells were counted and diluted to 2×10^5 viable cells per ml. Test or control sera were added to aliquots of the cell suspension at a 1:64 dilution. $[^3H]$thymidine was added to give a final concentration of 0.5 microCuries per ml. 200 microliters of the suspensions were placed in Corning 25860 Cell Wells plates. Ten replicate wells were used for each suspension. Controls consisted of MEM with no added serum, non-tumor bearing, and tumor bearing mouse serum. The test serum was obtained 3 days after removal of the primary tumor.

The plates were incubated at 37°, 100% humidity, 5% CO_2. At the end of the 24 hour incubation period the cells were harvested on Whatman MicroFibre filters using a Brandel Cell Harvester. The wells were washed with distilled water and then exposed for approximately 2 minutes to 0.25% Pronase in Gey's balanced salt solution. Distilled water washing was continued until the wells were clear of cells. The filters were dried and each pad was placed in a vial containing 5 ml of Aquasol. Activity was counted in a Packard Tricarb Liquid Scintillation Counter. The data are reported as counts per minute or as percent change from a control activity.

Conditioned Medium: An in-vitro growing cell line was obtained from C3HeB/FeJ transplantable primary tumors. The initial plating medium, removed after four days of growth, was discarded. The conditioned medium was collected from dishes showing a confluent monolayer of cells during the second week of cell growth.

The protein in conditioned or normal medium was precipitated by $(NH4)_2SO_4$ using 20, 30, 50, and 80% final saturation. The precipitated proteins were pelleted by centrifuging at 5000g for 45 minutes. Each fraction of protein was de-salted by dializing against distilled H_2O using membranes with 6-8,000 or 12-14,000 MW cut-off limits. The resulting slurry of proteins was lyophilized. The protein concentration in each fraction was determined by the Lowry method and ranged between 92-98%. Tumor bearing animals were injected with 350µg protein dissolved in 1 ml PBS or with PBS only. Heat inactivation of protein was accomplished by heating the PBS-protein mixture in a water bath at 56°C for 30 minutes or 100°C for 10 minutes.

RESULTS

Primary Tumor Removal on LI of Metastases in Six Different Tumor Models: After removal of a primary C3H, MXT_a or MC54 tumor, those with an LI ≥15% resulted in an increase in the LI of the residual tumor, which was evident at 24 hours.

Both the degree of increase and its duration varied with the tumor examined (Figure 1). Tumors with an LI <15%, MXT_b, 3LL and CD8 also demonstrated a significantly increased LI (P=0.01), but of a much lesser magnitude, particularly in the CD8 tumor.

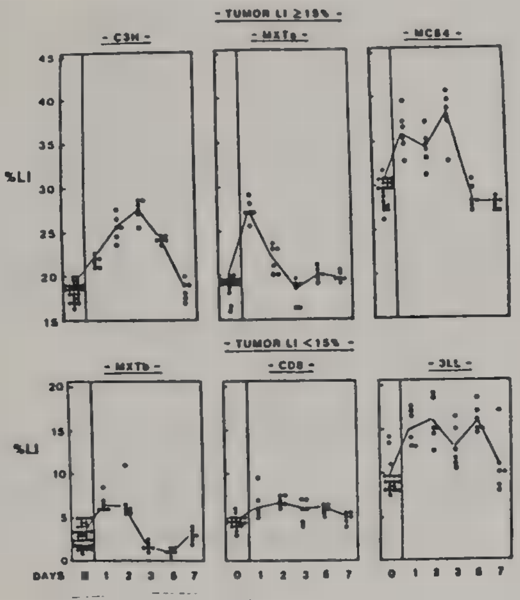

Figure 1

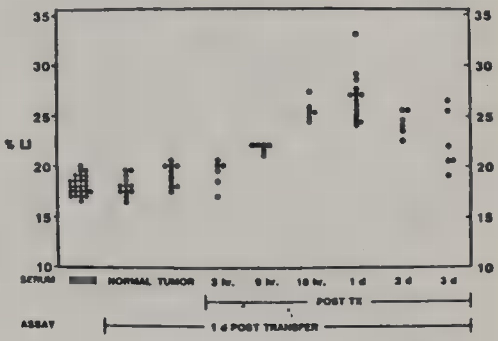

Figure 2

Fig.1 Efect of removal of mouse tumors with LI ≥15% on LI of residual tumor over time. Each dot in all figures represents LI in a single mouse.
Fig.2 Effect of the interval between removal of a C3H tumor and serum collection on LI of the recipient C3H tumor.
None = no serum, normal = no tumor, tumor = no removal.

Transfer of Serum Obtained Following Tumor Removal: In C3H tumor-bearing mice, transfer of serum from non-tumor-bearing mice following leg amputation, or from mice with the tumor in place, had no effect on the tumor LI (Figure 2), nor did serum obtained three hours after TX. As the time was increased, so was the LI of the recipient tumor, with the maximum effect at 18 and 24 hours, and a lesser effect at two and three days after TX. Regardless of whether the serum was obtained from mice with a single tumor removed or from those with a distant tumor focus remaining, the increased LI was the same (25% in both cases versus 18% in the control). In mice bearing two C3H tumors, one 244cmm and the other 69cmm in volume, the LI was similar in both (19% and 20%). Following transfer of TX serum, however, no response was observed in the large tumor, and the increase in the small one was less than that observed when only a single small tumor was present (Figure 3).

Multiple doses of serum on successive days failed to produce a greater increase than did a single dose (Figure 4). If the second transfer was delayed for seven days, however, when the LI had returned to the control value, the recipient tumor LI was equivalent to that produced by the first transfer.

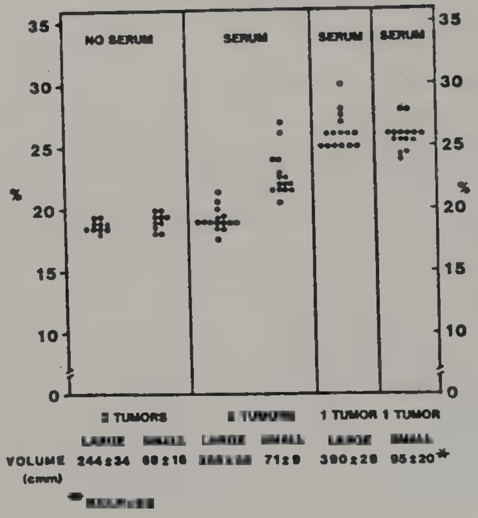

Figure 3

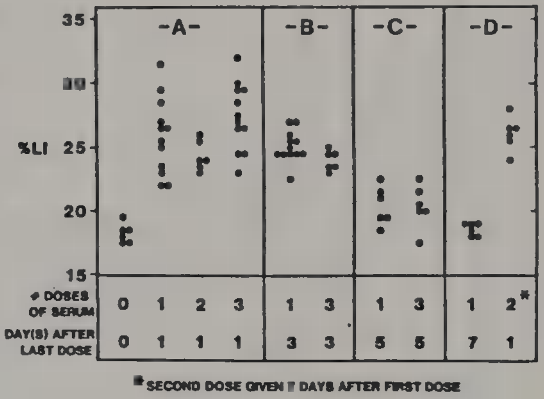

Figure 4

Fig.3 Effect of serum obtained one day following C3H tumor removal and transferred to recipients bearing one or two C3H tumors of different sized on the labelling index of the recipient tumors.
Fig.4 Effect of the number of injections, and the interval between the last injection and determination of the labelling index of C3H tumors in recipient; all serum was derived from donors 24 hours following C3H tumor removal.

The LI-stimulating effect of TX serum was not confined to the C3H tumor system but was found in varying degrees in the other five as well, with minimal effect on the CD8 tumor (Figure 5). Serum obtained following tumor removal was not specific for the host tumor but caused an elevation of LI in the other tumors employed (Figure 6). The exception was the CD8 tumor, which did not respond to C3H, MXT_a or MC54 tumor TX serum.

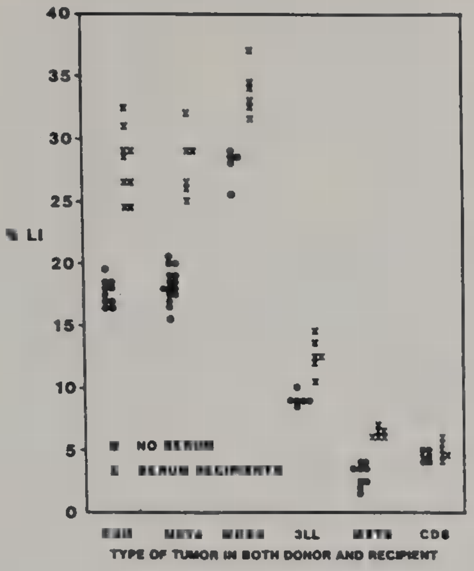

Figure 5

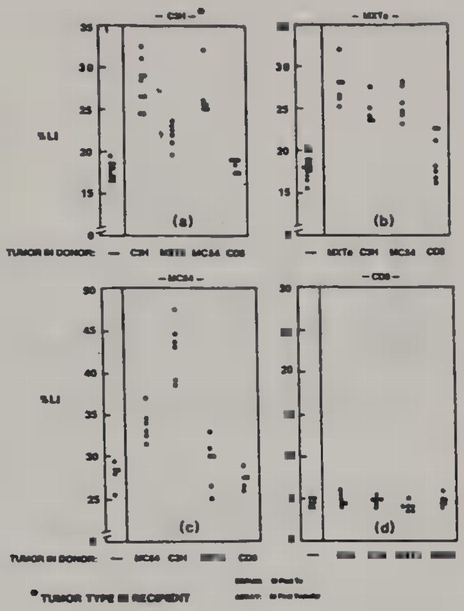

Figure 6

Fig.5 Transfer of serum obtained 24 hours following removal of tumors of various types to recipients with a tumor of the same type as in the serum donor: labelling index (LI) of recipient tumor 24 hours after transfer.
Fig.6 Transfer of serum obtained 24 hours following removal of tumors of various types to recipients with a tumor of a different type than in the serum donor: labelling index (LI) of recipient tumor 24 hours after transfer.

Incubation of C3H tumor cells in medium containing serum obtained after C3H tumor removal resulted in an increase in DNA synthesis greater than that observed when the serum was obtained from normal mice or from those with the tumor unremoved (Figure 7).

Transfer of Conditioned Medium: Inoculation of various ammonium sulfate precipitated fractions of medium conditioned by growth of the C3H tumor into mice bearing that tumor produced an increase in LI of tumors 24 hours later. The greatest effect was produced by the 30% fraction where the LI was 37% vs 19% in mice receiving nonconditioned protein or none (Figure 8).

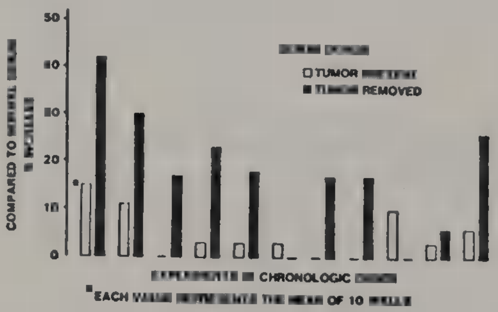

Figure 7

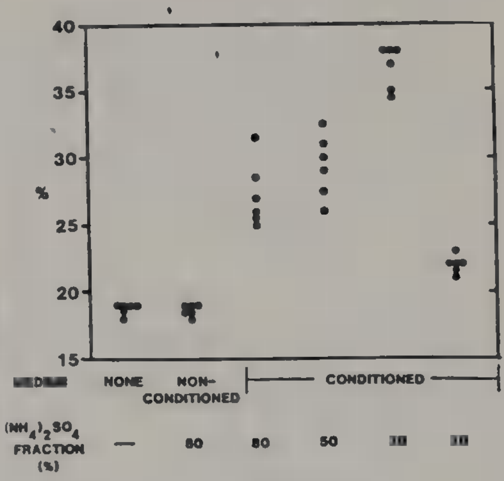

Figure 8

Fig.7 Stimulation of DNA synthesis in vitro by serum from C3H mice bearing a tumor or following tumor revoval: both compared to DNA uptake in the presence of serum from a nontumor-bearing mouse.
Fig.8 Presence of a growth factor in conditioned medium: effect on LI of C3H tumor in recipients.

Treatment of Host Prior to Tumor Removal: Administration of a single dose of CY five days before operation resulted in a marked decrease in LI to 6% instead of 18%. Following tumor removal, the LI in the residual tumor focus gradually returned to the control value. The 44% increase in LI of the residual tumor in untreated mice was completely prevented. Feeding TAM or implanting a Z depot prior to tumor removal was equally effective in preventing the kinetic change observed after operation (Figure 9,10).

Treatment of serum donors in the same manner eliminated the increase in recipient tumor LI that occurred when serum from untreated operated animals was employed. Serum from non-tumor-bearing mice fed TAM failed to affect the recipient tumor LI (not shown), indicating that the inhibition was not due to TAM or its metabolites in the serum.

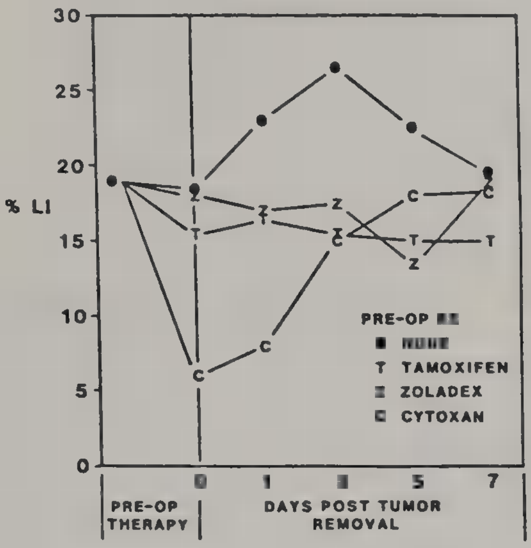

Figure 9

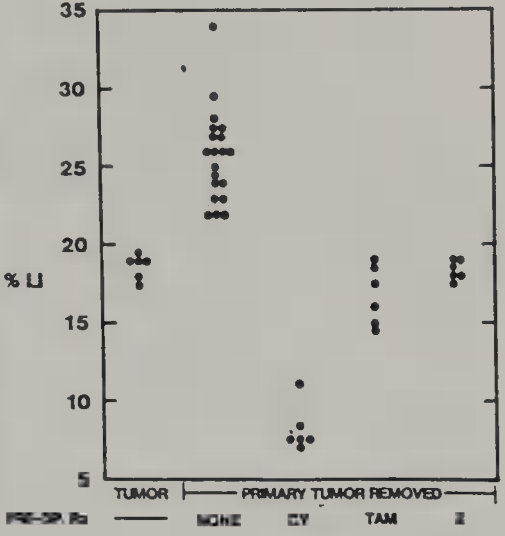

Figure 10

Fig.9 Effect of treatment of mice with cyclophosphamate (CY), tamoxifen or Zoladex prior to revoval of a C3H mouse tumor on the labelling index (LI) of a residual tumor focus over time.
Fig.10 Effect of treatment of mice with cyclophosphamide (CY), tamoxifen or Zoladex prior to the removal of a C3H tumor on the labelling index (LI) of a residual tumor focus of individual mice after 24 hours.

Radiation of the primary tumor (RTX) in the serum donor also abrogated the increase in LI of the recipient tumor (Figure 11).

Pretreatment of the Serum Recipient: Transfer of serum from untreated animals obtained following primary tumor removal to recipients treated with CY, TAM or Z failed to increase the tumor LI, as did transfer to untreated recipients (Figure 12). Irradiation of the recipient tumor also prevented any increase in LI.

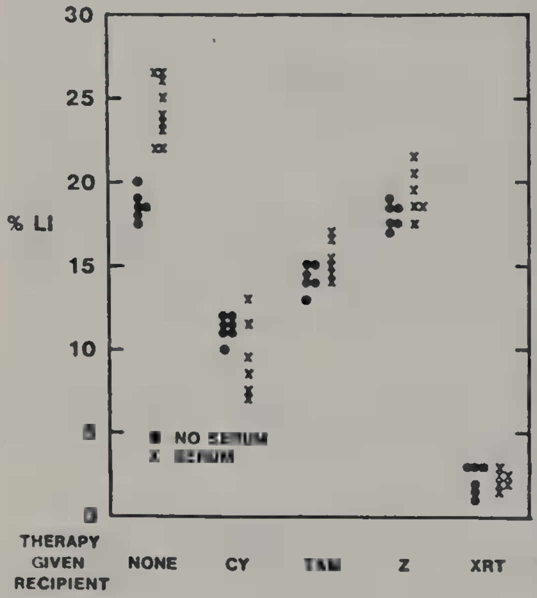

Figure 11

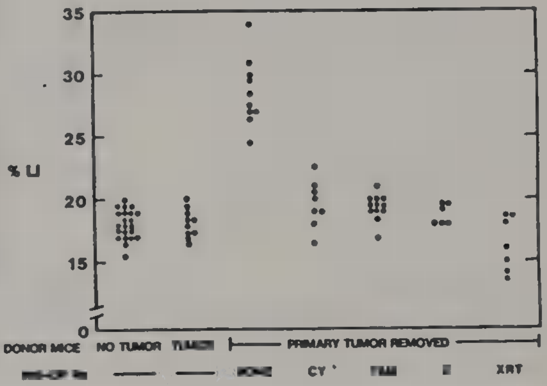

Figure 12

Fig.11 Effect of serum from donors treated with cyclophosphamide (CY), tamoxifen (TAM), Zoladex (Z), or irradiation (XRT) prior to tumor removal on the labelling index (LI) of recipient tumor. Animals receiving no preoperative therapy are designated as "none". Fig.12 Effect of serum obtained from mice following tumor removal on the labelling index (LI) of tumors in recipients treated with CY, TAM, Z, or XRT prior to serum transfer.

Discussion

The findings indicate that, in all six of the tumor models investigated, removal of a primary tumor produced an increase in LI of the residual tumor focus despite the variability of the histologic nature of the tumor, the strain of mouse employed or the number of cells undergoing DNA synthesis. The results demonstrate that the changes observed are related to a factor in the serum which promotes DNA synthesis in a distant metastatic tumor focus. Moreover, the findings refute the premise that removal of a primary tumor is a local phenomenon with no other biological consequences.

These observations resulted in speculation as to their mechanism(s). Operative and anesthetic trauma was ruled out. Suitable experimental controls have provided no evidence to support the concern that kinetic changes observed may reflect ordinary fluctuation of the LI during that period of tumor growth. Results of investigations support the premise that a growth factor rather than an inhibitor is responsible.

The observation that multiple doses of serum failed to enhance the increase in LI observed following a single dose suggests that only a limited population of tumor cells is capable of responding to the stimulus. The findings demonstrating no augmentation of LI following multiple doses of serum indicate that there is likely to be a finite population of cells in the G_0-G_1 phase that is capable of responding to the stimulating factor and of being transferred into more actively cycling cells.

Of interest were the observations obtained indicating that there is no specificity of growth factor which is related to the origin of tumors (virally or chemically induced) or to the strain of mice in which the tumors are grown. The sole specificity we have as yet observed is that only tumors producing a stimulating growth factor are capable of responding to it.

We hypothesize that the growth factor released by a tumor is in an "inactive" form and over time becomes "active." We also consider that, in a tumor-bearing animal, the rate of tumor cells traversing from G_0 to G_1 may be synchronized with the amount of the active component available, and that the active component is rapidly utilized. Investigation is required to determine whether the serum factor acts directly on tumor cells or modulates a host response which is responsible.

Studies were conducted to determine whether the production of the stimulating factor could be prevented by preoperative therapy and/or whether the treatments employed would inhibit the response to the factor. From the findings presented it appears that both functions are impaired. Serum obtained from animals which had received CY, TAM, Z or XRT failed to alter the tumor LI in untreated recipients bearing an isologous tumor. Furthermore, the failure of tumors in treated recipients to respond to serum obtained from untreated donors following tumor removal indicates that there is an altered response of the recipient tumors the growth-stimulating factor as a result of the treatment.

Consideration has been given to how the various therapies used might impair both the production and the response to the stimulating factor. We had concluded from previously obtained data that tumors producing the factor are those which are capable of responding to it. The current findings tend to support that conclusion.

Preoperative systemic therapy, sometimes referred to as "neoadjuvant" therapy, has been used in the treatment of patients with certain solid tumors[10,11,13]. Its use has often resulted in a decrease in tumor size permitting less extensive surgery. No randomized trials, however, have been conducted to determine whether such treatment more effectively controls micrometastatic disease than does the same therapy administered after operation. Until recently there has been no clear biologic rationale to justify the conducting of clinical trials to answer that question. With the present findings indicating that chemotherapy, tamoxifen, or radiation therapy given prior to operation prevents the increase in such cell proliferation, there is provided a basis for conducting trials to determine the worth of preoperative systemic therapy. Appropriate preoperative therapy should prevent cell proliferation following tumor removal and, consequently, prevent an increase in the number of resistant cells in the metastatic population.

There is no assurance that similar kinetic changes observed in animal models occur following removal of tumors in the human and that the temporal pattern of the kinetic changes (should they occur) is similar; nor is there certainty that the Goldie-Coldman hypothesis has relevance to patients who have tumors which by the time of diagnosis have been present for months to years[7]. The evaluation of preoperation therapy in the clinical setting is justified. A trial is currently being carried out by the National Surgical Adjuvant Breast and Bowel Project.

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REGULATION OF GROWTH IN NORMAL AND NEOPLASTIC CELL POPULATIONS BY A TISSUE SIZER MECHANISM: THERAPEUTIC IMPLICATIONS

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INTRODUCTION

Mammalian tissues, whether normal or neoplastic, usually exhibit deceleratory rather than exponential growth (Mayneord, 1932; Schrek, 1936; Klein & Revesz, 1952; Patt & Blackford, 1954; von Bertallanfy, 1957; Laird, 1964, 1969; McCredie et al., 1965; Simpson-Herren & Lloyd, 1970; Skehan, 1984 & 1986). This deceleration is characterized by a specific growth rate (SGR) that decays progressively with time. Most investigators have assumed that tumor growth deceleration results from the development of vascular insufficiency or host immunity (Mottram and Russ, 1906; Schrek, 1936; Thomlinson and Gray, 1955; Cataland et al., 1962; McCredie et al., 1965; Summers, 1966; Tannock, 1968, Lala, 1977). However, normal tissues also exhibit growth kinetics that are predominantly deceleratory. In a wide variety of normal vertebrate and invertebrate growth processes, deceleration results from a regulatory process of tissue sizing, a negative feedback process in which a tissue's growth rate is determined by its momentary size rather than by its chronological age or its initial innoculum size (von Bertallanfy, 1957; Rose, 1957; Weiss and Kavanau, 1957; Tanner, 1963, 1981; Bullough, 1965; Goss, 1969; Snow, 1981; Stebbing, 1981).

In this report we: review growth rate patterns of a number of normal and tumor cell populations; compare data on normal and regenerative growth of rat liver to that of a transplantable rat hepatoma of varying innoculum sizes; explore the role of tissue sizing in tumor growth regulation; and examine implications of the concept to the design of cancer therapy.

Kinetics of Growth Analysis

Many studies of growth have appeared in the literature. With few exceptions, analysis of these data has been restricted to equations of state (size as a function of time). State analysis tells little about underlying mechanisms, and is poorly suited to extracting quantitative growth parameters of biological significance. Velocity analysis of growth has been rare despite its considerable power to reveal reaction mechanisms. We therefore decided to apply velocity analysis to a number of published sets of growth data. Literature data bases were identified which presented growth measurements as a function of time. Most of the original data were presented in graphical form. Quantitative size and time values were obtained from xerographic copies of these published figures. From this data, specific growth rates were computer calculated. Specific growth rate (SGR) was calculated from the following equation:

$$SGR = 100\ (-1+2^{(tu)(Ln(S2/S1)/(0.69315)(tobs))})$$

where S1 and S2 are successive tumor size measurements, tobs is the interval between the measurements, and tu is the unit period of time (i.e. 1 day or 1 hour). The specific growth rate calculated by this equation has the units of percent increase in size per unit time.

Specific Growth Rate Patterns in Normal and Tumor Tissues

Skehan (1984) found that normal tissues exhibited several kinetic patterns of growth. Pure deceleratory growth was the most common. Growth was fastest at the earliest developmental age examined, and slowed gradually but progressively with time. A less common pattern consisted of a brief acceleratory phase followed by a much longer period of deceleratory growth. A smaller proportion of tissues and tumors exhibited either deceleratory or acceleratory-deceleratory patterns which ended in a prolonged phase of very slow terminal basal growth. In a few instances there was a prolonged deceleratory phase ending in a period of limited tissue regression which presumably reflects compensation for an overshoot of the tissue's set point for final adult size. Fewer than 10% of the data bases possessed an exponential phase. Exponential growth in nature appears to be uncommon (Skehan & Friedman, 1984; Skehan, 1986). Regardless of the particular growth pattern a tissue exhibited, the deceleratory was consistently the most prominent phase of growth.

The normal data bases did not permit the tissue sizing hypothesis to be tested. In all of the data bases, SGR decreased monotonically. This is compatible with but does not prove the hypothesis of growth regulation by tissue sizers. Since there is no evidence that the growth deceleration of normal tissues results from the development of vascular insufficiency, and since the deceleratory phase generally extends from early embryogenesis or birth into early adulthood, it is likely that deceleration results from tissue sizing in many of the instances.

Of the 122 normal data bases, 104 had a deceleratory phase that exhibited downward convexity when SGR was plotted against size (Tab. 1). Downward convexity indicates that a unit increase in size exerts a stronger

TABLE 1. Summary of Deceleratory Phase Curvature of Specific Growth Rate Plots of Normal and Tumor Data Bases (from Skehan, 1986)

	N	Downward convexity	Linearity	S shaped	Downward concavity	Indeterminate
Normal Tissues	122	104	4	7	2	5
Tumor	126	110	5	0	2	9

growth inhibitory effect on a small tissue than on a large one. Its practical implication is that most of the total growth inhibition to which a tissue is eventually subjected develops early in a tissue's growth while it is still quite small in size. Opposite curvature (downward concavity) indicates that little growth inhibition occurs until a tissue is quite large and approaching its final adult size. This pattern of growth was observed in only two of the 122 normal data bases. A linear decay of growth rate with size was observed in four cases, and an S-shaped decay in seven.

Tumor growth was also predominantly or exclusively deceleratory. Of the 126 tumor data bases, all contained a deceleratory phase. Fifty exhibited pure deceleratory kinetics at all observed times, 23 had an acceleratory-deceleratory pattern, nine began with a brief exponential phase that was followed by a deceleratory phase, 27 exhibited deceleratory growth followed by a terminal phase of slow basal growth, and 15 exhibited an acceleratory-deceleratory-terminal basal pattern. Clearly the predominant mode of growth for tumors, as for normal tissues, was deceleratory.

Nine of the tumor data bases provided growth data for innocula of more than one size, and therefore permitted a test of the tissue sizing hypothesis. Of the nine, six exhibited classical tissue sizing while one did not; the remaining two were equally compatible with both tissue sizing and age dependent growth regulation, so that no conclusion could be made about the nature of their control. Thus the growth of at least six and possibly eight of the nine tumors demonstrate a growth pattern suggestive of growth regulation by tissue sizers.

As with normal tissues, the great majority of tumor deceleratory phases exhibited downward convexity in their SGR-size plots (Tab. 1). Most of the growth inhibition to which the tumors were ultimately subjected was imposed early in their development while they were still small.

Comparison of Normal, Regenerative and Neoplastic Growth

The predominant characteristics of in vivo growth of normal and neoplastic tissues are summarized as follows:

Growth is predominantly deceleratory; deceleration is probably caused by tissue sizing in many instances; most of a tissues's growth inhibition is imposed early in its development when it is still quite small (downward convexity of SGR-size plots); and inverse Nth root equations provide the best family of growth models, Nth power equations the worst (Skehan 1984, 1986).

As another way of judging if normal and tumor growth demonstrate similar or different patterns of growth we have analyzed a normal and a tumor data base which are matched with the corresponding normal tissue of origin from the same species. The respective specific tissue of tumor origin and species for this comparison were liver and rat.

Figure 1 illustrates analyses of postnatal and regenerative growth of the rat liver. From the top down in figure 1 we see: liver mass plotted against time, the specific growth rate plotted against time, the specific growth rate plotted against liver mass and the rate of cell proliferation (tritiated thymidine labeling index one hour after isotope injection) plotted against liver mass.

Postnatal liver growth, as measured in mass, shows a deceleratory growth rate pattern indicative of growth regulation by tissue sizing. The plot of labeling index vs. mass (bottom of Fig. 1) shows a pattern of acceleration of growth followed by a deceleratory phase which demonstrates downward convexity of slope. The demonstration of an early acceleratory growth phase in the labeling index data may be accounted for by the earlier sampling times in this data set compared to other data sets or the greater sensitivity of the labeling index to growth rate change.

The surgical removal of two thirds of the liver mass in young adult rats results in a rapid regeneration of the remaining liver. The remaining liver returns to its original size in 48 hours. The growth rate time and size curves both show a pattern of acceleration followed by a deceleratory phase with downward convexity. The rate of cell proliferation as estimated by labeling index exhibits the same pattern of growth rate acceleration followed by a deceleratory phase showing downward convexity. The

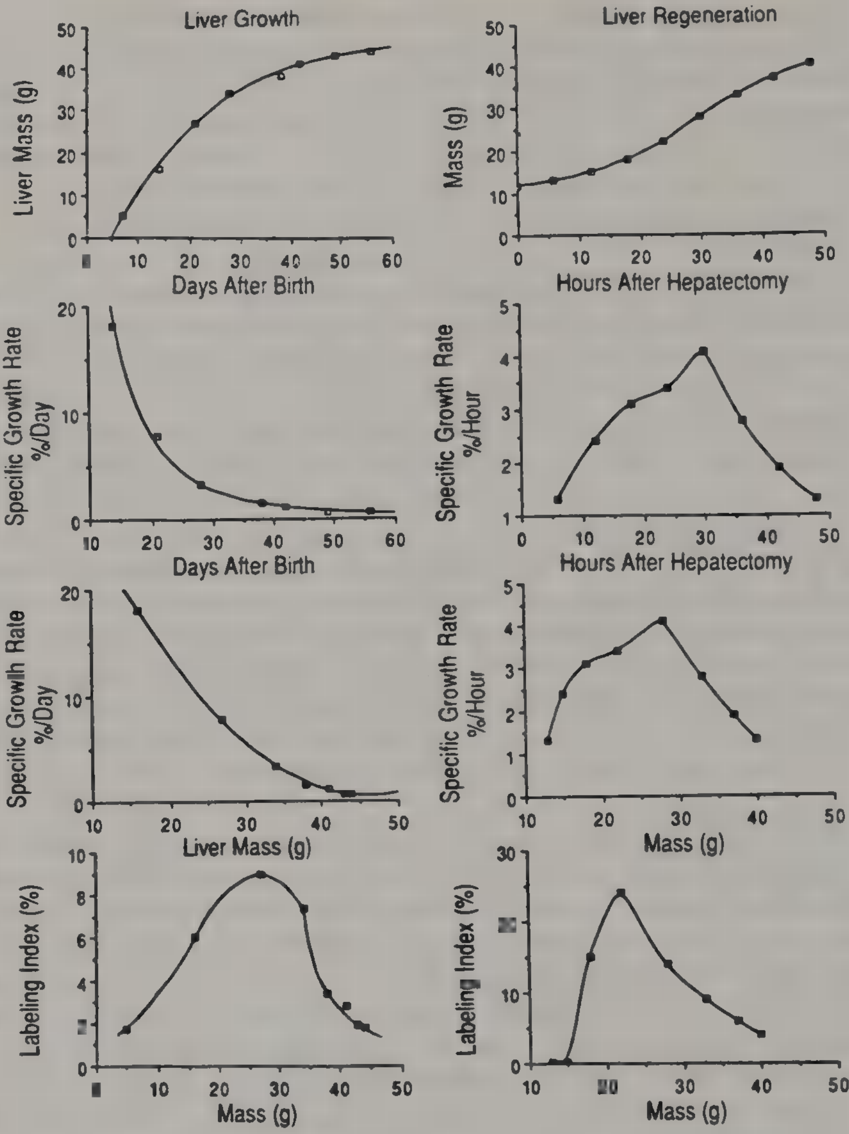

Figure 1. *In vivo* growth kinetics of postnatal and regenerative normal rat liver (original data bases from Grisham, 1969). From top down the graphs report: growth in mass with time; the specific growth rate (% change per day or hr) with time; specific growth rate as a function of mass; and the pulsed tritiated thymidine labeling index (an indicator of the specific growth rate) as a function of mass. Two-thirds of the liver mass was surgically removed from adult rats in the liver regeneration studies.

labeling index begins to decelerate four hours prior to the specific growth rate in mass suggesting that changes in genome replication precede changes in mass growth during deceleration.

The data suggest the possibility that postnatal and regenerative rat liver growth are both negatively feedback regulated by [illegible] tissue sizing mechanism. Interestingly, Grisham (1969) and others have provided experimental evidence for a humoral factor which demonstrates negative feedback properties to rat liver growth.

To test whether a rat liver-derived tumor also demonstrated growth regulatory tissue sizing, different amounts of tumor mass were subcutaneously innoculated into rat hosts. If a tissue sizer is operating, the subsequent growth of these innocula will be a function of their momentary size only and not of their chronological age, state of development, or innoculation size. We have been able to test this by analyses of an excellent set of data generously provided to us by William B. Looney of the University of Virginia at Charlottesville.

Figure 2 shows tumor growth curves for Hepatoma 3924A innoculated as varied amounts of tumor mince into ACI rats. Tumor volume in cc from individual rats was calculated by: one half length x width x height measurements. Thirteen rats were followed in each innoculation size group. Only the mean tumor size for each group was plotted and used in the analyses. Figure 2a shows measurement of state as a function of time for the tumors. The growth curves were sigmoidal. The curves showed a "lag" period during which little growth appeared to occur. However, inspection of the rate curves (Figure 2b) showed this "lag" to be an artifact. It was actually the period of fastest growth, but was obscured by the inability of rectilinear plots to display large proportional changes in small values of state.

Figure 2b illustrates how tumor specific growth rates changed with time. Growth was fastest at the earliest time examined, and decayed progressively thereafter. The SGR values fell along a set of descending curves, suggesting that the growth of this tumor may be temporarily regulated or programmed.

Figure 2

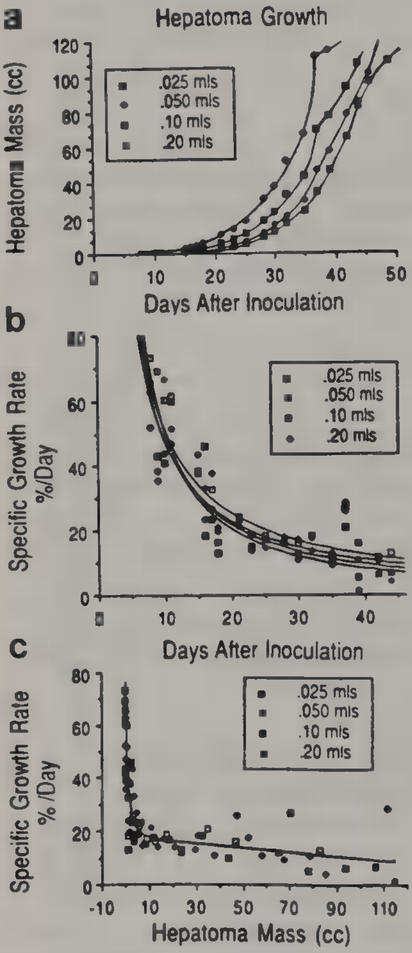

Figure 3

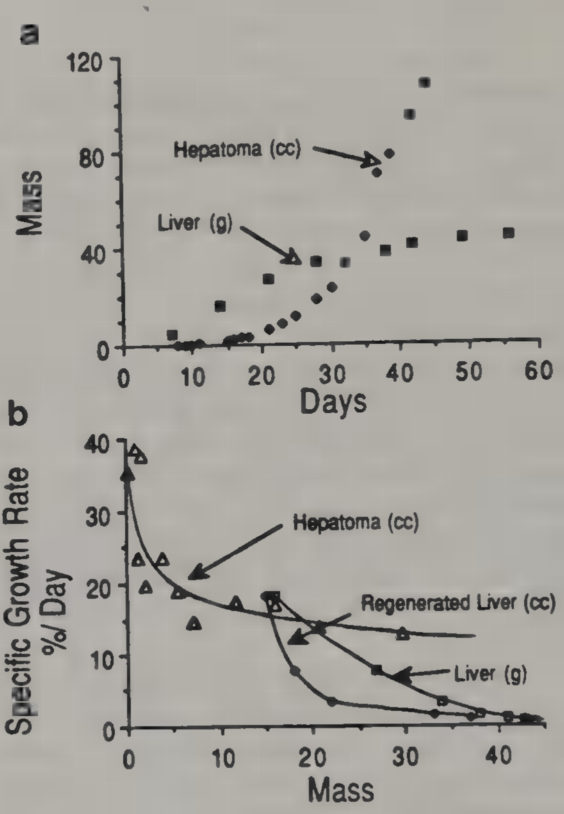

Figure 2. In vivo growth kinetics of a transplantable hepatoma 3924A innoculated into ACI rats (original data bases generously provided by William B. Looney of the University of Virginia at Charlottesville). Each symbol represents a different innoculum size. The data from 13 rats are averaged for each data point. From top down the graphs report: growth in mass with time, the specific growth rate (% change per day) with time and the specific growth rate as a function of tumor mass.

Figure 3. Comparison of rat liver and rat hepatoma (3924A) growth kinetics. The top graph is a plot of mass in either g or cc as a function of time. The bottom graph is a plot of specific growth rate as a function of time (data from figures 1 and 2).

Figure 2c illustrates how specific growth rate changed with tumor size. Rate-size plots for each set of hepatoma data each generated superimposable curves in which the specific growth rate was a monotonically decreasing function of momentary size but was independent of both innoculum size and chronological age. These are the characteristics of a growth process regulated by tissue sizing.

Data in Figure 3a and b allows direct comparison of the rat liver and rat hepatoma growth assuming a cc of tumor volume weighs one gram. Mass plotted against time (Fig. 3a) shows the hepatoma to obtain a larger size in a shorter time than liver. Overall the terminal hepatoma size is about twice that of the liver. The same data sets as well as the specific growth rate of the regenerating liver (data in fig. 1) are each plotted in Figure 3b. All three exhibited the same qualitative growth pattern of deceleratory proliferation with downward convexity. They differed, however in their quantitative dependencies on tissue size (Fig. 3b). This lack of superimposability indicates either that the three tissues respond differently to a single growth inhibitor or that their tissue sizers have different recognitive determinants and are therefore regulated by different growth inhibitors.

A search of published tumor growth data bases revealed two that appear not to demontrate a growth pattern suggestive of tissue sizing (Fig. 4a & b). The most carefully studied exception is the mouse Ridgway Osteogenic Sarcoma (ROS). Figure 4 shows a graph of the labeling index of this tumor plotted against its mass. Growth of the ROS tumor was first acceleratory but did not markedly decelerate as the tumor size increased. In fact the cell proliferation rate remained remarkably high throughout the growth of the ROS tumor. Examination of the morphology of this ROS tumor suggests possible reasons that can be used to explain its growth pattern. Simpson-Herren (1975, 1977) points out that the ROS implants (100 mg) initially decrease in size for 6 days, at which time the 26 mg tumor has a rim of viable tissue three to four cells wide surrounding a necrotic core with no viable cells. A tissue shrinking in size will be driven out of SGR-size equilibrium. An increase in SGR is needed to restore equilibrium, i.e., acceleratory growth.

If birth rate and death rate reach and maintain equal values, then (live) tissue size is constant and, by the contact equilibrium hypothesis of tissue sizing (Skehan and Friedman 1982) will produce exponential growth and therefore a constant labeling index. As the tumor grows (0.1-0.5g) it progresses through a highly cellular and well vascularized phase (illustrated in Fig. 4a). With increasing size the ROS tumors show cords of viable cells around capillaries within an increasing core of acellular necrotic material. Thus the failure of this ROS tumor to demonstrate a marked deceleration in labeling index with increasing tumor size may well be due to the fact that the measured size of the tumor includes an exceptionally large proportion of noncellular necrotic material. The high labeling index can therefore be accounted for by a high rate of cell birth that is balanced by a high rate of cell death with the accumulation of noncellular necrotic material. Given that tissue sizing results from feedback inhibition by a viable tissue mass, a correction for the disproportionately large necrotic mass may well show this ROS not to be an exception to the mass inhibition rule of growth regulation. The rapid cell loss and accumulation of necrotic mass suggest that this tumor has not accumulated enough viable tissue to demonstrate a deceleratory growth phase. The acceleratory growth rate of the Fortner plasmacytoma (Figure 4b) may have a similar explanation. However this tumor eventually did show a deceleratory phase suggesting it has accumulated enough viable tumor mass to demonstrate tissue sizing.

These two apparent exceptions to tissue sizing of tumor growth call to attention potential problems in the universal use of tumor size uncorrected for necrotic mass to evaluate the regulation of tumor growth, as Lala has previously reported (1977).

Interaction Between Multiple Tumors of the Same Origin in the Same Host

Few investigators have carefully examined interactions between multiple tumors of the same origin. This subject is critical to the decision of whether or not to therapeutically remove or irradiate a primary tumor mass. If a small tumor is growth inhibited through tissue

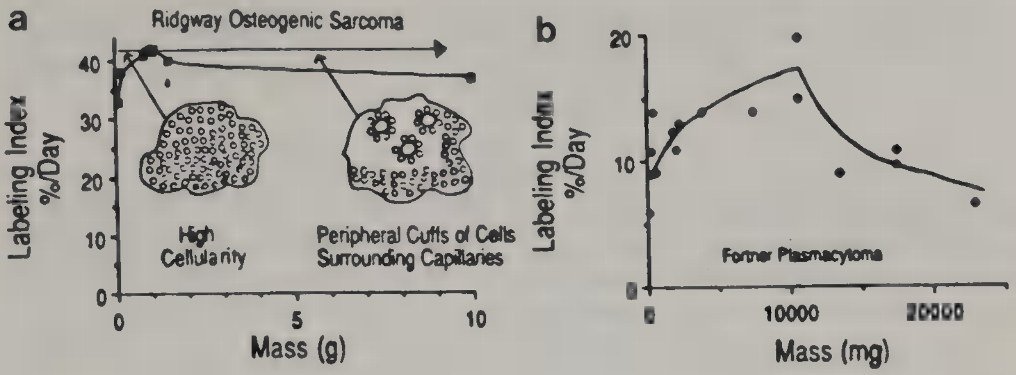

Figure 4. Graphs of the *in vivo* tritiated thymidine pulse labeling index plotted as a function of tumor mass for the transplantable Ridgway Osteogenic Sarcoma (ROS) in mice and the Fortner plasmacytoma in hamster. Drawings representing ROS tumor morphology at two stages of tumor growth are included on the graph (original data bases from Simpson-Herren et al., 1970, 1974, 1977). Each ROS data point for the labeling index is the average from five mice.

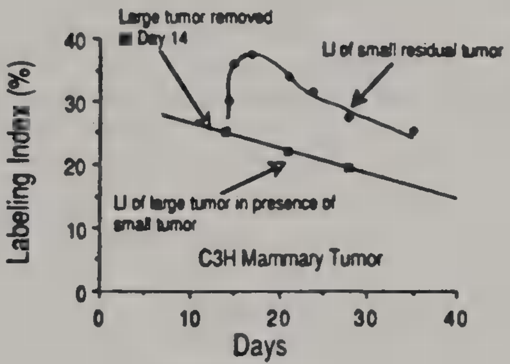

Figure 5. *In vivo* growth kinetics of a large and small tumor of the same origin in the same host. Graph of the temporal change in labeling index of a large C3H mammary cell tumor in one leg of a mouse and the labeling index of a small C3H mammary cell tumor in the other leg following removal of the large tumor (original data sets from Gunduz et al., 1979).

sizing by a larger tumor in the same host animal, then removal of the larger tumor should stimulate the growth of smaller tumors at distant sites. One experimental data set allowed examination of this question.

Gunduz et al., 1979 transplanted large innocula of C3H mammary tumor cells into one leg of mice and a small innocula into the other leg. This procedure generated two tumors of different sizes in the same mouse. The labeling index of the large tumor was followed with time after tumor transplantation in one group of mice. In another group of such mice the large tumor was removed at 14 days after tumor inoculation. The labeling index of the small tumor was followed at times after removal of the large tumor. Figure 5 graphs the results of this experiment. The labeling index of the large tumor decreased with time. Removal of the large tumor on day 14 resulted in a rapid acceleratory increase in the labeling index followed by a deceleratory phase with downward convexity. We interpret these results to indicate that the small tumor demonstrated feedback inhibition from the total tumor mass of both tumors combined. Table 2 summarizes these same surgical removal data on the labeling index as well as the results of a similar experiment where the main (large) tumor was subjected to X-irradiation instead of surgical removal. The data suggest that removal or irradiation of the main tumor leads to stimulation in growth of the remaining small tumor.

Another set of data related to the interactions between multiple tumors in the same host comes from the work of DeWys (1972). His tumor model was the transplantable Lewis lung carcinoma. When transplanted subcutaneously into C57BL/6 mice this tumor grows at the site of inoculation but in six days post-innoculation metastatic nodules are also found in the lungs. The mass of the primary and the metastatic lung tumors were followed in mice killed at different times after tumor transplantation. Figure 6 shows a plot of the SGR of the primary tumor and the lung tumor nodules as a function of total tumor mass (sum of the primary and lung nodules). The deceleratory growth pattern indicates tissue sizer inhibition characteristics. If growth of the lung nodules is under inhibition from total tumor mass, one would expect the labeling index of the metastatic tumors to show the same fit as the primary tumor. As shown in Figure 6

TABLE 2. Effect of Primary Tumor Radiation (RTX) or Removal on Labeling Index of a Distant Small Tumor[a,b] (Fisher et al., 1986)

Days post treatment	Sham RTX	RTX[a]	Large tumor removal
0	17.6	17.6	17.8
1	17.8	16.6↓	25.5↑
3	17.2	19.8↑	26.3↑
7	17.7	15.5↓	18.9

[a] All but the large tumor was shielded from 50 Gy from a 100 kV x-ray machine over 31 min in C3HeB/FeJ mice with a transplanted C3H mammary adenocarcinoma. The small tumor was in the opposite leg.

[b] Arrows indicate a significant increase (↑) or decrease (↓) from zero time values

the SGR data from lung nodules rapidly fall to the SGR of the large primary tumor data. Thus growth of the metastatic nodules does appear to be subject to inhibition from the primary tumor and does display tissue sizer characteristics.

Therapeutic Implications

Tissue sizing appears to govern the in vivo growth of many normal tissues and tumors. That tumor growth can be governed by tissue sizing has three important implications: (1) tumor growth can be highly regulated; (2) this regulation can be accomplished by the same kinetic control mechanism that regulates the growth of many normal tissues; and (3) growth regulation per se may not be the central lesion in some neoplastic diseases.

Tissue sizing results from a growth inhibitory negative feedback commmunication between member cells

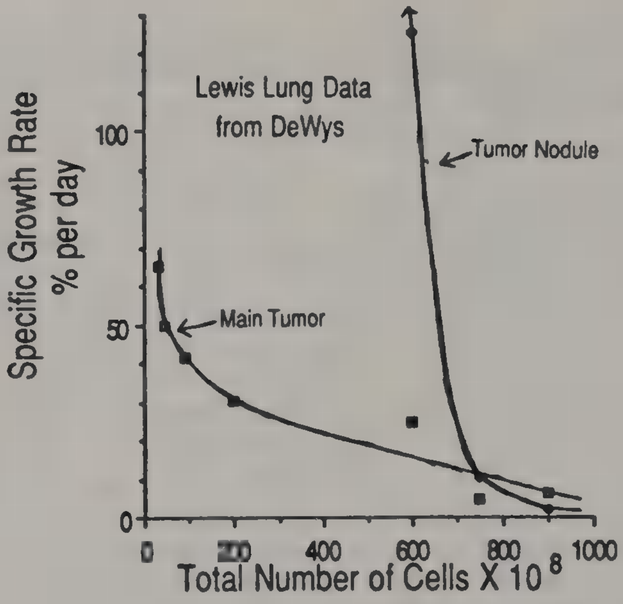

Figure 6. *In vivo* specific growth rate (SGR) of Lewis lung carcinoma in leg (main tumor) and in both lungs (tumor nodule). Mean tumor data points were taken from chart 1 of DeWys (1972). The SGR of the metastatic lung nodules rapidly decreases to that of the main leg tumor.

within a multicellular community. The signals which mediate this communication appear to exhibit a degree of target tissue specificity that effectively constitutes a cellular recognition mechanism. Available evidence indicates that certain of these signals are highly cell-type specific, others influence multiple cell types, and that homotypic interactions are often more effective than heterotypic interactions at exerting inhibition (Abercrombie et al., 1968; Eagle et al., 1968; Njeuma, 1971a, 1971b; Weiss & Njeuma, 1970; Skehan and Friedman, 1974; Steck et al, 1979; Yaoi, 1984; Kinders and Johnson, 1981; Skehan, 1984). It would therefore appear that deceleratory growth mediated by tissue sizers involves two separate steps. The first is a cellular recognition event which acts as a switch to turn on the sizer, while the second is the actual operation of the sizers growth inhibition machinery per se. Sizing can presumably proceed only if an appropriate recognition has already taken place this suggests the possibility that neoplastic transformation may arise from an alteration in the surface recognition process which precedes tissue sizing. Some tumors, and perhaps some metastatic nodules as well, behave as if they were new types of tissues with unique surface recognitive determinants but normal growth regulatory machinery. Thus in some instances neoplastic transformation may be a process of tissue neogenesis.

Tissue sizing has several important therapeutic implications. Most of the clinical antitumor drugs in current use are antiproliferatives which act by killing rapidly growing cells. Systems governed by tissue sizers grow rapidly only when they are small. Tumors whose growth is sizer regulated should therefore be extremely sensitive to antiproliferative drugs when they are very small in size. This provides a powerful theoretical argument for the use of adjuvant antiproliferative chemotherapy following the reduction of tumor burden by resection or irradiation if the metastatic tumor cells are still under inhibition by the primary tumor.

By the time such a tumor reaches a clinically detectable size, however, antiproliferatives are likely to be counterproductive as a primary therapy because of their comparative ineffectuality against slowly growing large tumors and their strong toxicity toward rapidly proliferating host tissues. Most human tumors first become clinically detectable at 1-10 cc. Even at a size of 1 cc most animal model tumors are slowly growing and unlikely to respond well to antiproliferative drugs. Thus tumors regulated by growth inhibitory tissue sizing may present a serious chemotherapeutic management problem because of their development of antiproliferative resistance. This suggest that even modest improvements in early detection may lead to sizable improvements in the efficacy of antiproliferative therapy.

Tissue sizing has another clinically important implication. Any reduction of tumor mass which does not eliminate all neoplastic disease can lead to an increase in the growth rate of remaining tumor cells. Figure 4 suggests that where residual burden is small this increase can be explosive. With slowly growing nonaggressive tumors and with disseminated disease whose sizer inhibited metastases are located in sites potentially more dangerous than the primary, any reduction in tumor burden may actually reduce patient lifespan by triggering the explosive growth of remaining foci. However, where the metastatic tumor growth does not appear to be inhibited by the primary tumor then removal of the main tumor may not be expected to stimulate growth of the metastasized tumor. The problem is to know whether the metastatic tumor cells in the host are or are not under growth inhibition by the primary tumor mass before the main tumor is removed. This type of information is needed in the decision to use or not to use adjuvant chemotherapy with antiproliferatives.

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TUMOR DIFFERENCES, DRUG DIFFERENCES, TREATMENT DESIGN DIFFERENCES, AND EFFECTS ON THERAPEUTIC OUTCOME (DEGREE AND DURATION OF THERAPEUTIC RESPONSE)

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INTRODUCTION

Cancer chemotherapy when used alone or in an adjuvant setting in animals or humans always includes (1) a cancer of some age, size, stem cell death rate, growth rate, degree of cellular heterogeneity and spread, (2) a host, (3) a drug or drugs, and (4) critical decisions with respect to drug selection and the method of delivery.

Because these variables are important in planning treatment, I often feel the urge to try to relate things we think we know about these things in plots and word tables--along with necessary reservations (e.g., see Figures 1-5 and Tables 1-5). And then I wonder why the reservations are necessary.

In December of 1988, I wrote a long informal report in which this "seemingly impossible" question was posed: Why do different cancers with different growth rates and patterns respond so differently to different drugs and combinations of drugs delivered in the same and different ways?

There can be little doubt that the true answers to this multifaceted question lie in these broad areas: (1) tumor differences, (2) drug differences, and (3) treatment design differences. My major reason for writing the above-mentioned progress report was to see how far I could get without drowning in reservations, exceptions, or inconsistencies--within and between hard data and presently accepted theory. Another was that I wanted to plan future analyses, searching for correlations (or inconsistencies) that might have been overlooked in earlier interpretations of therapeutic response results.

This paper will have to do with (a) an alternative interpretation of the basic phenomenon underlying the correlation between tumor growth rate and responsiveness to chemotherapy, (b) important independent effects of dose intensity and total dose on the degree and duration of therapeutic response, and (c) some gentle hints or suggestions to those who plan and interpret the results of chemotherapeutic trials.

ON INTERPRETATION OF THE UNDERLYING REASONS FOR A CORRELATION BETWEEN THE GROWTH RATE OF CANCERS AND THEIR RESPONSIVENESS TO CHEMOTHERAPY

Long ago I thought I saw a correlation between the growth rate of experimental cancers and their responsiveness to the then available anticancer drugs. This deduction was based on growth rate curves such as are presented in Figure 5 and results observed in many chemotherapeutic trials. As far as I can see, there was nothing wrong with this early deduction, but the interpretation isn't as simple as I once may have thought.

One thing that seems to be constant in most cancers is that they are constantly changing in a total burden sense, in a kinetic or growth rate sense as a result of tumor stem death, in a genetic and biochemical sense as a result of mutations of T/0 to T/R cells, and in an anatomic sense as a result of metastasis. To make it worse, the rate of change of these changes varies in individual tumors of a given type, and even more so between different cancers.

In the late 1960's, I thought that a major reason for asymptotic growth curves (decreasing growth rate with increase in tumor size) was increasing proportions of tumor stem cells entering a resting state for longer periods. At that time we had observed that resting leukemia cells were almost completely resistant to very high concentrations of S-phase specific drugs like ara-C, methotrexate, or 6-thioguanine. Later we observed that resting leukemia cells (T/0 cells) most certainly were not resistant to alkylating agents or DNA binders, if they subsequently attempted DNA replication and division in the absence of drugs.

Today when I look at growth rate curves like those in Figure 5, recalling the degree and duration of response of different neoplasms to drugs at different stages of advancement, I am reminded of the following:

1. The deduction of Steel (1977) that the decrease in tumor growth rate with increase in size is largely a reflection of an increase in tumor stem cell death rate,
2. the conceptual model of Goldie and Coldman (1983), and
3. consistent observations we have made over many years (see comments in the body and footnote of Table 1 and entries 2 and 3 in Table 2).

Briefly, the conceptual model of Goldie and Coldman offers an alternative explanation for the correlation between tumor growth rate and responsiveness or lack of responsiveness to chemotherapy (see below).

		Dominant Phenomenon Affecting:	
	Concept	Decreasing Growth Rate With Increase in Size	Increasing Resistance to Chemotherapy
1.	Late 1960's	Resting T/0 Cells	Resting T/0 Cells
2.	Above Model of G. & C. (1983)*	Increasing tumor stem cell death rate	Increasing proportion and absolute number of limiting T/R cells

* Presuming that T/0 cells mutate to T/R cells only while they are replicating their DNA in preparation for division. And, the higher the stem cell death rate, the higher the number of DNA replications and divisions required to reach a given tumor size.

ON THE INDEPENDENT EFFECTS OF DOSE INTENSITY AND TOTAL DOSE ON THERAPEUTIC OUTCOME (DEGREE AND DURATION OF THERAPEUTIC RESPONSE)

During 1987 and 1988 it seemed that I was working full time for Dr. Hryniuk, trying to respond to another broad and elusive question: Are chemotherapeutic trial results available in animal model systems that might allow one to assess the importance of dose intensity--independent of the effects of total dose--on therapeutic outcome? (Hryniuk, 1988).

My initial response to this question was that I wasn't sure, even though we had carried out hundreds of multi-armed trials in animal tumor systems in which the doses and schedules of different drugs were manipulated in all sorts of ways. The reason for the initial uncertainty was that we never analyzed large bodies of observed data in the precise context of his question.

One thing we could be certain about was this: There is a consistent direct relationship between the individual dose level, dose intensity, and total dose of a particular drug and both the degree and duration of response of a particular cancer--as long as the staging and schedule are constant and lethal toxicity or limiting T/R cells do not intervene.

This is a restatement of the old "dose-response law"--with four necessary reservations appended. But this old law doesn't answer Dr. Hryniuk's question. To answer his question we must ask and answer another broad question: Is there a direct relation between dose intensity and total dose on both degree and duration of therapeutic response when the treatment schedule is manipulated in many ways? The answer to this question is an emphatic "No" based on extensive data examined in considerable detail (see comments under "treatment design" in Table 1, entry 7 in Table 2, and the implications of the plot in Figure 3 which are consistent with observed results). (Skipper, 1988).

In fact, I wasted time at the beginning of these analyses before it became apparent that (a) one cannot identify independent effects of dose intensity and total dose on therapeutic outcome by comparing results from trials in which the schedule was kept constant, and (b) the effects of dose intensity and total dose on the degree and duration of therapeutic response are not equatable in a quantitative sense when the treatment schedules are quite different. I mention my own mistakes in the hope that it might help others avoid them in the future.

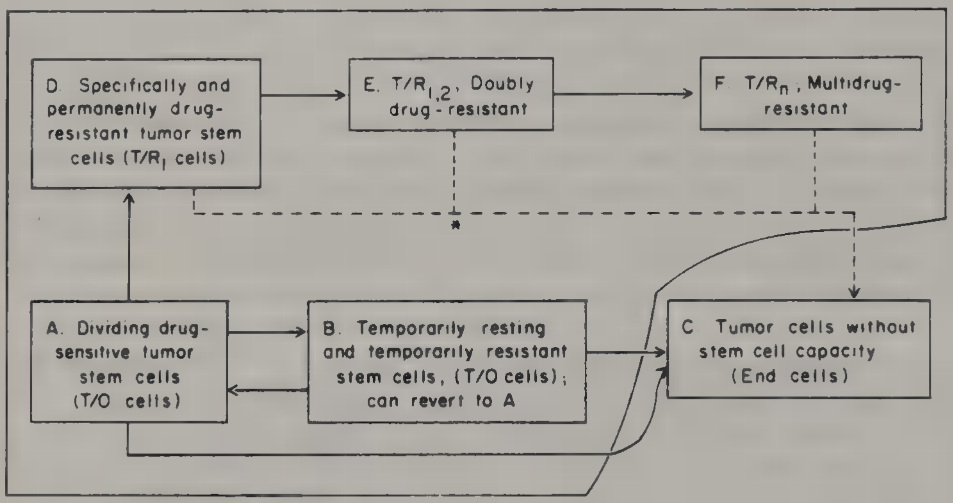
SOME GENTLE HINTS OR SUGGESTIONS HAVING TO DO WITH THE FUTURE

Based on retrospective analyses carried out over the past two years, I will gently offer some suggestions to those who plan and interpret (a) chemotherapeutic trials with animal tumors in animals or human tumors in animals, (b) clinical trials where drugs are used alone or in an adjuvant setting, and (c) *in vitro* experiments where cancer cells are exposed to different drug concentrations for different periods and then assayed for surviving fraction.

Be careful to bear in mind that (1) the two major types of therapeutic response endpoints (degree and duration of response) often are *not* equatable in a quantitative sense, (2) failure to consider the effects of limiting T/R cells on both degree and duration of response may lead to serious misinterpretations (and to new trials that will not be helpful with respect to practice or theory), (3) temporary minimum responses or PR's may underestimate the degree of tumor stem cell reduction, and (4) there are important independent effects of dose intensity (dose rate) and total dose on toxicity and on the degree and duration of therapeutic response when drugs are used alone or in an adjuvant setting.

Today there are rational approaches to improving treatment of disseminated cancers, but we need better guidance in their application at the human level. To achieve better guidance, I believe it may be necessary to reexamine and, in some instances, reinterpret clinical trial results, focusing on the impact of tumor differences, drug differences, and treatment design differences on therapeutic outcome.

Finally, one measure of clinical progress in the years ahead might be the rate of change in the fraction of patients (bearing a particular cancer) that can be treated with the realistic goal of cure *versus* the fraction for which palliation is the best that can be hoped for. In experimental cancers, the best treatment design for cure often is quite different from the best design for palliation.


D. Specifically and permanently drug-resistant tumor stem cells (T/R$_1$ cells)
E. T/R$_{1,2}$, Doubly drug-resistant
F. T/R$_n$, Multidrug-resistant
*
A. Dividing drug-sensitive tumor stem cells (T/O cells)
B. Temporarily resting and temporarily resistant stem cells, (T/O cells); can revert to A
C. Tumor cells without stem cell capacity (End cells)

Tumor Stem Cell Compartments

*Resistant phenotypes may go into and out of resting phase but they remain T/R$_1$ or T/R$_{1,2}$ or T/R$_n$ cells.

Figure 1. Tumor stem cell heterogeneity which increases with size and stem cell death rate and decrease in growth rate. (1) Resting T/0 cells are stem cells that temporarily are not replicating their DNA in preparation for division. While resting they are very refractory to S-phase specific drugs but not to alkylating agents or DNA binders. (2) Limiting T/R cells are stem cells that are specifically and permanently resistant to a drug or drugs for quite different reasons. (3) The most frequent single cause of chemotherapeutic failure in experimental cancers is the presence or emergence of limiting T/R cells. (Of course, there are others such as "barriers" to exposure of tumor cells to drugs in a $\underline{C}$ with $\underline{t}$ sense.)

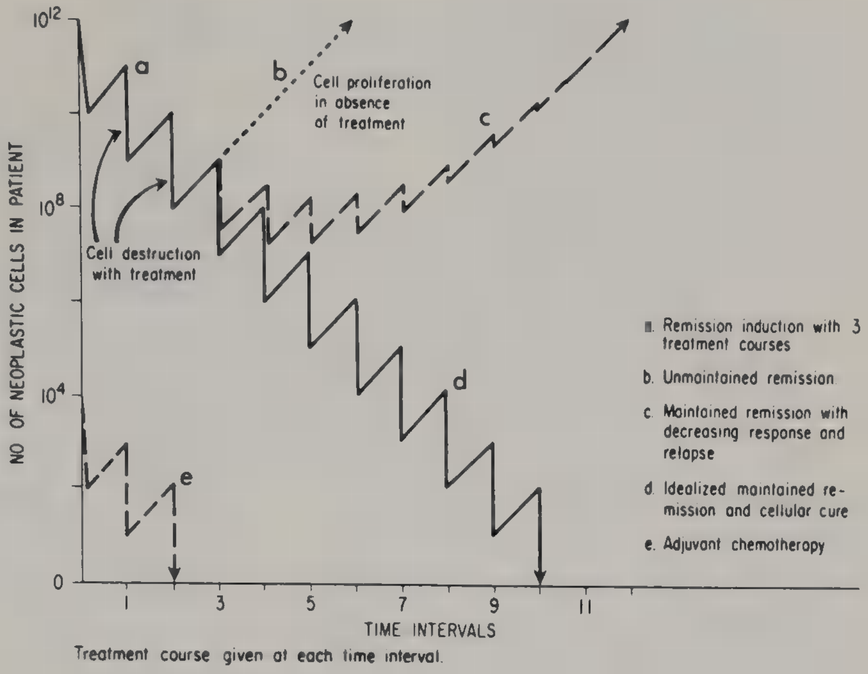


Figure 2. Idealized time-action response curves for the total tumor stem cells. Curve ab: Cessation of treatment after three courses. A second response with the same drug or drugs might or might not be possible, depending on the T/R to T/0 stem cell ratio at the cessation of treatment and at relapse. Curve ac: A classical illustration of tumor response followed by tumor progression during continuing undiminished treatment with the same drug or combination of drugs. A nadir will be reached when the surviving tumor stem cell burden is comprised of about 50% limiting T/R cells (T/R to T/0 ratio = 1.0). Tumor progression will be observed when the T/R to T/0 ratio exceeds 1.0. Curve ad: This type of curve would almost never be expected on treating individuals bearing, say, 10^9 or > tumor stem cells with a single drug because of the high probability of the presence of T/R cells with specific resistance. Burkitt's lymphoma and choriocarcinoma may or may not be exceptions to this generalization. Curve e: Cure of relatively small numbers of metastatic stem cells after local surgery or radiation that left no T/R cells that were resistant to the drug or drugs employed.

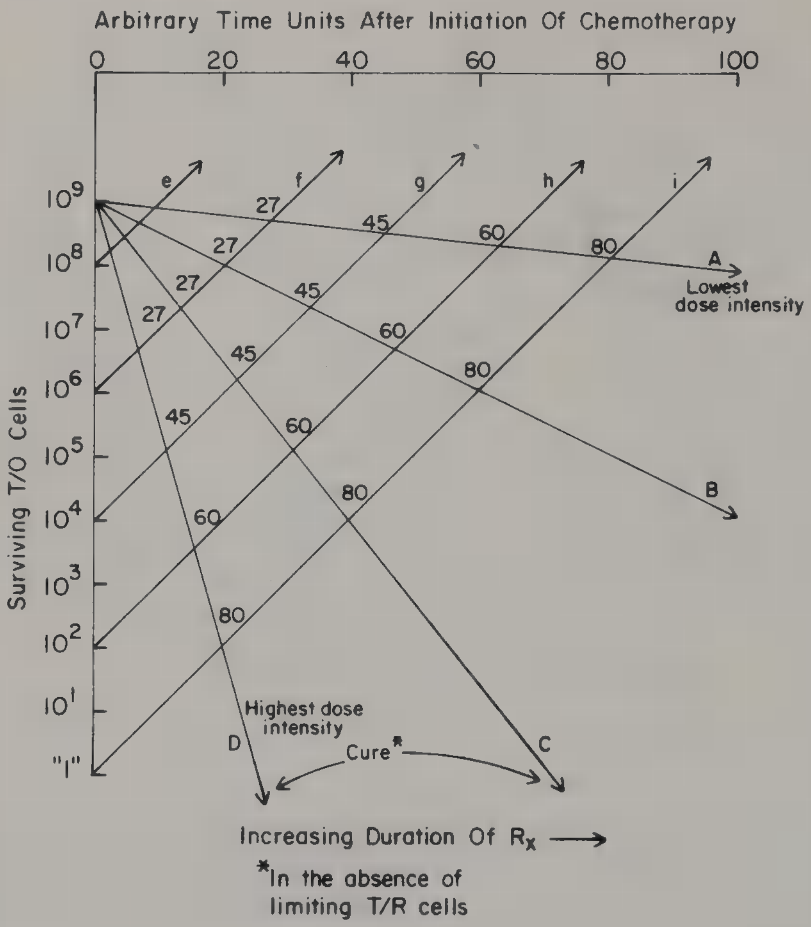


Figure 3. An idealized illustration of the effects of dose intensity, duration of treatment, and total dose on the degree and duration of therapeutic response (ignoring limiting T/R cells). (1) Lines A, B, C, and D represent increases in dose intensity and, in turn, increases in the net rate of T/0 cell kill. (2) Lines e, f, g, h, and i represent assumed regrowth rates after cessation of treatment at the points where these lines cross lines A, B, C, and D. (3) The numbers along lines A, B, C, and D are total doses calculated by multiplying the dose intensity (mg/kg/arbitrary time unit) by the duration of treatment. (4) The implication intended is that we should expect dose intensity to be the dominant variable with respect to the degree of response at the nadir and total dose to correlate best with duration of response of treatment failures.

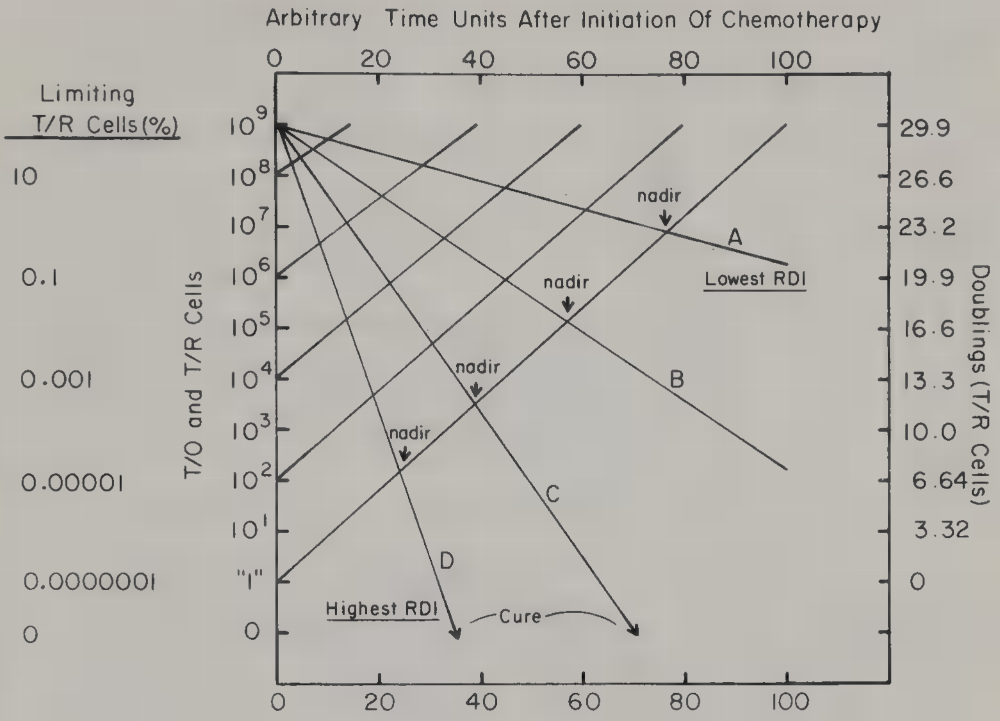


Figure 4. An idealized illustration of the effects of dose intensity, duration of treatment, total dose, and the proportion of limiting T/R cells on the degree and duration of therapeutic response. (1) The nadir will be reached when the surviving tumor stem cell mix is about 50% T/0 and 50% T/R cells. (2) Note the effects of increasing dose intensity, duration of treatment, and total dose on the degree and duration of therapeutic response (as in Figure 3 where T/R cells were ignored).

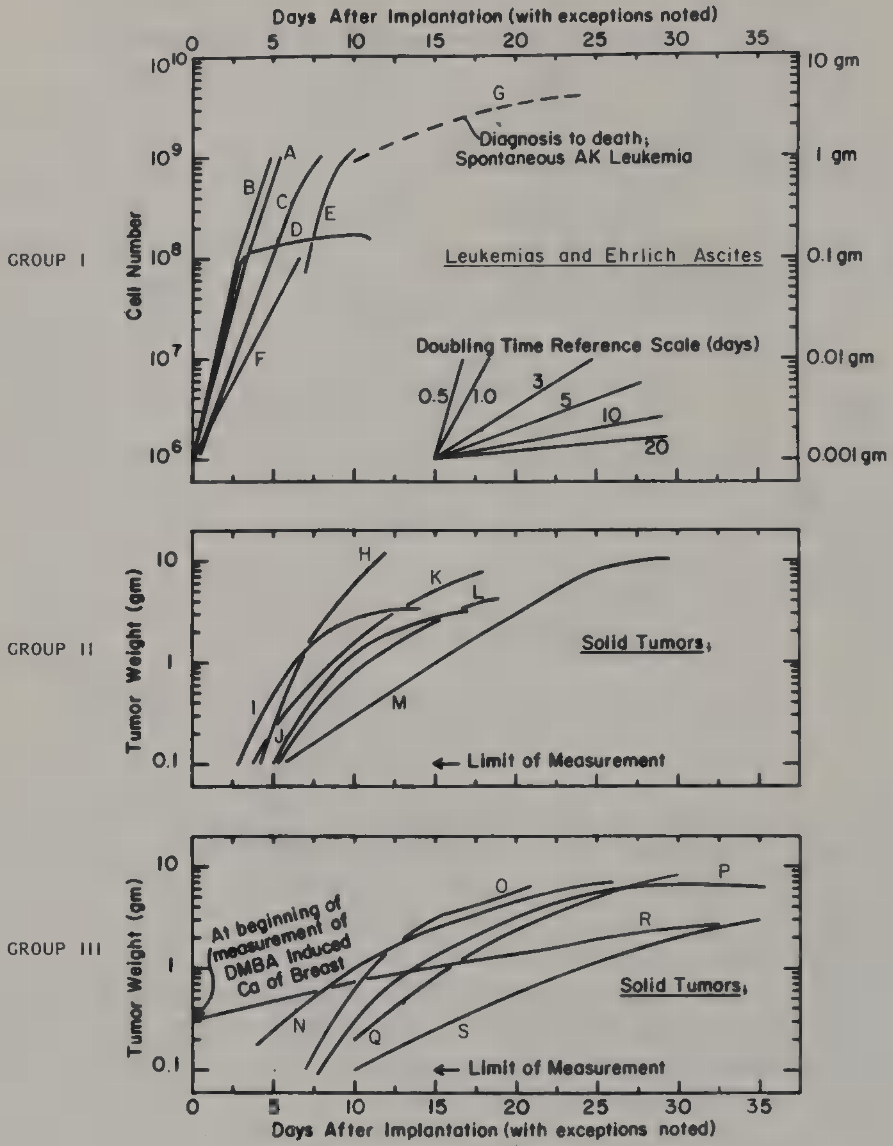


Figure 5. Growth rates of experimental neoplasms that may be classified as responsive, moderately refractory, or very refractory to chemotherapy. Note: When the growth rate of any of these neoplasms becomes notably asymptotic, you can almost bet your boots they contain limiting T/R cells.

TABLE 1. Why Do Different Cancers With Different Growth Rates and Patterns Respond so Differently to Different Drugs and Combinations of Drugs Delivered in the Same and Different Ways?*

Tumor Differences	Drug Differences	Treatment Design Differences
Normal cells of origin and burden and spread at the initiation of treatment. Kinetics: Growth rates and the rate of change of growth rates with increase in tumor age, size, and stem cell death rate. (Increases in stem cell death rate increase the number of DNA replications and divisions required to reach a given tumor size and, in turn, the chances for dividing T/0 cells to mutate to limiting T/R cells.) Genetics: The invariable increase in the probability of the emergence of drug-resistant phenotypes (T/R cells) with increase in tumor age and size (local and metastatic). The invariable inverse relation between the tumor stem cell burden and curability by drugs or combinations of drugs. Barriers to exposure of tumor stem cells to drugs in a C with t context. Others: Including intrinsic drug resistance and significant response of some cancers to natural or stimulated host-immune factors or systems.	Mechanisms of Action: Most anticancer drugs kill cancer (and normal) stem cells by effects on DNA replication or functions or cell division. S-phase specific drugs do not kill cells that are not replicating their DNA in preparation for division during exposure to cytotoxic concentrations (t is more critical than C). This is not true for alkylating agents and DNA binders like adriamycin or actinomycin D. Mechanisms of T/R cell resistance to drugs: These vary with the class of drug and their mechanism of action at the biochemical level. The degree of resistance of diverse T/R cells selected by antimetabolites may be as high as 1,000-fold, but almost never is as high as 10-fold in T/R cells selected by alkylating agents. Biologic half-life of the cytotoxic forms of the drug in a C with t and C x t context. Toxicity: Differences in the degree of toxicity to different normal stem cells. There are profound differences in effect of schedule on the toxicity of S-phase specific drugs like ara-C, MTX, or 6-TG, compared with alkylating agents or DNA binders.	Dose and Schedule: 1. Individual dose level 2. Interval between doses 3. Number of doses 4. Duration of Rx (2 x 3) 5. Total dose (1 x 3) 6. Dose intensity $\frac{(1 \times 3)}{(2 \times 3)}$ Although dose intensity and total dose are not independent dosage units, they do have important independent effects on both toxicity and therapeutic outcome. The independent effects cannot be identified when the schedule is constant. Dose intensity is the dominant variable with respect to cure or nearness to cure at the nadir; total dose correlates best with the duration of response of treatment failures (i.e., the duration of temporary PR's and CR's and the survival time of treatment failures--see Figure 3). These relationships seem to apply for all sorts of drugs and neoplasms--as long as toxicity or limiting T/R cells do not intervene (see Figure 4).

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* The answers to this seemingly "impossible" question almost surely lie in one or more of the three broad areas indicated. Specific answers as to which, when, why, and how frequently must come from careful analyses and cautious interpretation. (The pitfalls on the path to valid conclusions based on inductive reasoning have been known to philosophers for over 2000 years.)

Note: Much of the data that have influenced my views on the question(s) in the table heading is contained in informal reports to the Division of Cancer Treatment of the National Cancer Institute (USA) written in 1987 and 1988. These retrospective analyses have led me to this conclusion: The increasing probability of the presence of limiting T/R cells with increase in tumor age and size is the single most frequent cause of chemotherapeutic failure when optimally selected and delivered drugs or combinations of drugs are used alone or in an adjuvant setting. Of course, there are others.

TABLE 2. Some Seemingly Consistent Concepts, Hypotheses, Theories, and "Laws" Having to do With Success and Failure in the Treatment of Disseminated Cancers

1. In order to achieve cure in disseminated cancers, it is necessary to eradicate the tumor stem cells (T/0 and T/R) in both the primary and metastatic sites. When host defense mechanisms (natural or stimulated) help, this is [redacted] bonus.

2. There is an invariable inverse relation between the total tumor stem cell burden and curability by chemotherapy, albeit with wide variations in the total burden curable by [redacted] given drug or combination of drugs in different cancers. Why is the reservation necessary?

3. There is a direct relation between the total tumor stem cell burden and the probability of the presence of limiting T/R cells, but with significant differences in this probability in different types of cancer at [redacted] given stage of advancement. (See the very helpful models of Goldie and Coldman and the somatic mutation theory of Luria and Delbrück (1943) with emphasis on the "fluctuation" in the numbers of phage-resistant phenotypes in similar-sized populations of E. coli cells and early fluctuation tests of Law (1952) in an experimental leukemia.)

4. Prior to treatment one may expect the following relative proportions of neoplastic stem cells in almost any type of cancer: $T/0 > T/R_1 > T/R_{1,2} > T/R_n$ (if any)--see Figure 1. This is easy to document in experimental neoplasms.

5. Since the origin of metastatic tumor cells is the "primary," one may expect zero metastatic tumor stem cells at the earliest or early stages. This is documentable by many years of successful treatment of varying fractions of many cancers (that metastasize at different rates) by local surgery or radiation. Unfortunately, some cancers are almost always widely metastatic when first detected.

   In cancers that already [redacted] metastatic at the time of local surgery or radiation, [redacted] may expect the [redacted] relative proportions of metastatic T/0 and T/R cells [redacted] indicated in 4 above. The first metastatic T/R cells (if any) may have [redacted] from the primary or from mutation of T/0 cells after becoming metastatic.

6. There is [redacted] direct relation between the dose intensity and toxicity of most anticancer drugs [redacted] long [redacted] the interval between doses and number of doses is constant. The tolerated dose intensity decreases rapidly with increase in the duration of treatment--to [redacted] point--and then decreases gradually. The interval between doses affects the shape of [redacted] curve in which dose intensity is plotted against the duration of treatment leading to a given degree of toxicity (e.g., the LD10 or LD50).

7. There is a consistent direct correlation between the individual dose level, dose intensity, and total dose of [redacted] effective drug and the degree and duration of response of [redacted] given neoplasm--as long as the schedule is constant and toxicity or limiting T/R cells do not intervene. Each of the above reservations is real and documentable. For example, if one pools therapeutic response results--regardless of

schedule--some of the correlations mentioned persist while others do not. With alkylating agents and DNA binders, dose intensity is the dominant variable with respect to the degree of therapeutic response (cure or nearness to cure at the nadir), but total dose often correlates best with the duration of response of treatment failures (the duration of temporary PR's and CR's and the survival time of treatment failures). With an S-phase specific drug like ara-C, another critical treatment variable is apparent; namely, the choice of an individual dose level, interval between doses, and treatment duration that will result in exposure of all neoplastic cells to cytotoxic levels of ara-C when they are in S-phase (i.e., replicating their DNA in preparation for division).

8. Combination chemotherapy when optimally selected and delivered is superior to single drug treatment because of its effects on both T/0 and limiting T/R cells.

9. Surgery plus combination chemotherapy is superior to surgery alone because surgery may reduce the total burden of T/0 and T/R cells to a point where combination chemotherapy will eradicate the residual metastatic cells or, failing that, significantly delay progression of the metastatic disease to a lethal burden.

Note: The above seem to me to be compatible with information gained in other areas (disciplines): Biochemistry, genetics, cell biology and kinetics, toxicology, pharmacology, pharmacodynamics and therapeutic design versus observed end results.

TABLE 3. Drug-Resistant Sublines of L1210 and P388 Leukemia Selected During the 1960's and 1970's for Study of Cross-Resistance and Lack of Cross-Resistance Between the Major Classes of Anticancer Drugs

L1210 Leukemia	P388 Leukemia
L1210/Methotrexate	P388/Methotrexate
/6-Mercaptopurine	/ara-C
/6-Thioguanine	/ara-A
/ara-C	/Azacytidine
/Hydroxyurea	/5-FU
/5-FU	/Cyclophosphamide
/Cyclophosphamide	/L-PAM
/L-PAM	/DON
/Phosphoramide mustard	/BCNU
/BCNU	/cis-DDPt
/cis-DDPt	/Vincristine
/BIC	/Adriamycin
/Adriamycin	/Act D
	/AMSA

Note: These sublines were selected by Schabel and associates and detailed results on cross-resistance and lack of cross-resistance may be found in their publications and informal reports from Southern Research Institute.

B16 Melanoma	Methyl CCNU	Single max. tolerated doses	B16 Melanoma/ methyl CCNU	The duration of response of animals bearing 10^5-10^7 SC implanted B16 melanoma cells was assessed by delay in appearance of measurable tumors compared with untreated controls (T-C). For previously untreated B16 cells, T-C was 15 days. After 2 or 3 passages in animals treated with a single MTD of methyl-CCNU, the T-C was essentially zero.

Note: These results were compiled by the author of this report from data obtained by my associates (Schabel, Griswold, Corbett, Laster, and Mayo) during the 1970's.

TABLE 4. Rate of Selection of Some Singly, Doubly, and Multidrug-resistant Sublines of L1210 Leukemia

	Parent Line	Drug or Drugs	Schedule	Number of Treated Passages Required to Select to Significant or Complete Resistance	Drug-resistant Sublines Selected
1.	L1210/0	MTX	qd (x 6)	2	L1210/MTX
2.	L1210/0	6-MP	qd (x 6)	1	L1210/6-MP
3.	L1210/0	6-TG	qd (x 6)	2	L1210/6-TG
4.	L1210/0	Ara-C	qd (x 6)	2	L1210/ara-C
	"	"	q3hr (x 8)	1	"
5.	L1210/0	PalmO-ara-C	Single dose	1	L1210/ara-C
	"	"	qd (x 9)	1	"
6.	L1210/0	Cyclophos.	Single dose	1-4	L1210/CPA
	"	"	qd (x 9)	2	"
7.	L1210/0	BCNU	Single dose	2	L1210/BCNU
8.	L1210/0	Ara-C + 6-TG	Simult., qd(x9)	1-2	L1210/6-TG/ara-C
9.	L1210/6-MP	PalmO-ara-C + MTX	"	1	L1210/6-MP/ara-C
10.	L1210/0	6-MP	Simultaneous, qd (x 6)	21	Resistant to the combination
		6-TG		9	Resistant to 6-MP or 6-TG
		6-MeMPR		9	Resistant to 6-MP, 6-TG & 6-MeMPR
		MTX		21	Resistant to above and ara-C
		5-FU		33	Resistant to above and 5-FU
		Ara-C		41	Resistant to all 6 drugs given singly
11.	L1210/0	6-MP	Each drug given once on succeeding days (6-MP first)	2	Resistant to combination
		6-TG		5	Resistant to 6-MP and 6-TG
		6-MeMPR		5	Resistant to 6-MP, 6-TG and 6-MeMPR
		MTX		13	Resistant to above and 5-FU
		5-FU		17-29	Resistant to above and ara-C
		ara-C		>60	Resistant to all 6 drugs given singly

(Continued)

12.	L1210/0	6-MP	Each drug given once on succeeding days (reverse order from above)	1-5	Resistant to the combination
		6-TG		5	Resistant to 6-MP and 6-TG
		6-MeMPR		5	Resistant to 6-MP, 6-TG and 6-MeMPR
		MTX		13	Resistant to above and 5-FU
		5-FU		41	Resistant to above and MTX
		ara-C		45	Resistant to all 6 drugs given singly

Note: The above experiments were carried out by D. J. Hutchison and F. Schmid of Sloan Kettering Institute and F. M. Schabel, Jr. and M. Trader of Southern Research Institute during the 1970's and were examined and tabulated by the author of this report.

In these experiments the leukemia cell burden in the first and subsequent treated passages was 10^6 or >. These and much additional data point to (a) the invariable inverse relation between leukemia cell burden and curability by single drugs and combinations of drugs, (b) the direct relation between the leukemia cell burden and the probability of the presence of limiting T/R cells, and (c) the importance of selecting the optimum dose intensity and method of delivery of each drug in a combination.

TABLE 5. Failure of Single Drugs and Combinations of Drugs in Treatment of Animals Bearing Different Solid Tumors (Due to Selection and Overgrowth of Limiting T/R Cells in the Original Host)

Neoplasm	Drug	Schedule	Drug-Resistant Subline Selected	Comments
Plasmacytoma (Pla-1)	Cyclophosphamide	Various max. tolerated repetitive dose regimens	Pla-1/CPA	High CR rates were achieved in animals bearing 1 gram tumors along with some cures. But the majority of animals suffered recurrence during continuing undiminished treatment with cyclophosphamide. These recurring tumors, when passed and retreated, proved to be resistant to cyclophosphamide.
Lewis Lung Carcinoma	Methyl CCNU	Two maximum tolerated doses given with a one-week interval between	LL/MeCCNU	PR's and CR's were achieved in animals bearing 0.5 gram tumors, but all recurred after the second dose. When the recurring tumors were harvested, passed, and retested for response to similar doses of methyl CCNU, they were almost completely resistant.

(Continued)

Ridgway Osteogenic Sarcoma	Cyclophosphamide	q14d (x 4)	ROS/CPA?	Two to three gram tumors regressed and recurred after MTD's of CPA alone. PR's were followed by recurrence during treatment with MTD's of 6-MP alone. An optimally delivered alternating combination of CPA and 6-MP was curative. CR's were followed by recurrence during alternating courses of CPA + 6-MP when dose intensity of the combination was too low.
	6-MP	Four qd(x11) courses	ROS/6-MP	
	CPA + 6-MP	Alternating schedule	None; or ROS/6-MP, or ROS/6-MP/CPA, depending on the RDI	
Colon Tumor 36	PalmO-ara-C (a slow release form of ara-C)	q7d (x 27)	Colon 36/ara-C	All animals bearing 0.2-0.3 gram tumors achieved CR's and all but two were cured. There were two recurrences during treatment and when harvested, passed, and retreated with palmO-ara-C, they indeed were resistant. When animals bearing 2-3 gram Colon 36 tumor were treated with repetitive doses of palmO-ara-C, PR's and CR's were observed, but essentially all animals suffered recurrence during continuing undiminished treatment.
Colon Tumor 36	A Triazine Antifol (NSC 127755)	Repetitive qd (x9) courses until death	Colon 36/Triazine Antifol	In animals bearing 0.5 gram tumors, PR's and CR's were achieved, but all recurred during continuing undiminished treatment.
Mam 16/C	Adriamycin	q7d (x 6)	Mam 16/C/Adr	PR's and CR's were achieved in animals bearing 0.2-0.3 gram Mam 16/C tumors, but all recurred during adriamycin treatment.
	CAF	Simultaneous; q7d (x 6)	Mam 16/C/Adr	PR's and CR's of longer duration were achieved, but all animals suffered recurrence during the last course of CAF. The recurring tumors were resistant to adriamycin, but not notably resistant to cyclophosphamide or 5-FU.
Colon Tumor 07/A	DTIC; Dimethyl triazenoimidazole carboxamide	Repetitive q2d(x5) courses over 2 months	Colon 07/A/DTIC	High CR rates of short duration were achieved but all tumors recurred during continuing undiminished treatment with DTIC. One of the recurring tumors was passed and retested for response to DTIC. It was resistant.

> Most of these sublines were selected by repeated treated passages, but in many instances this was not necessary. If the leukemia cell burden [redacted] 10^7 or > and treatment [redacted] continued long enough, drug-resistant sublines were selected in the original host and the animals died during continuing undiminished treatment with the same drug.
>
> The heritable biochemical changes responsible for the specific and permanent resistance of such mutant neoplastic stem cells to the drug that selected them (and others) are generally compatible with the mechanisms of action of the drugs at the biochemical level.

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GENETIC AND KINETIC FACTORS IN THE DRUG RESISTANCE OF MICROMETASTATIC DISEASE

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INTRODUCTION

Treatment failure associated with the adjuvant chemotherapy of malignancies such as breast cancer have been attributed to such factors as the presence of drug resistant clones of cells in the metastatic sites (Goldie and Coldman, 1979). or the presence of a proportion of clonogenic tumor cells that are temporarily nondividing (Steel and Lamerton, 1968). These two explanations are not necessarily mutually exclusive, but depending upon which phenomenon constitutes the predominant effect then this will dictate different chemotherapeutic strategies.

GENETIC FACTORS IN DRUG RESISTANCE

A great deal of evidence has been accumulated in recent years indicating that the presence of genetic drug resistant phenotypes constitutes an important factor in the treatment failure of advanced malignancies (Ling, 1982; Goldie and Coldman, 1984) Typically, this is associated with initial regression of the tumor followed by the resumption of growth in the face of chemotherapy that was initially effective in causing tumor cytoreduction. In many instances biochemical changes have been identified in these recurring tumor cells consistent with the cells acquiring resistance mechanisms that allow them to circumvent the effect of the antineoplastic agents (Hill, 1982). A particularly important category of such resistance mechanisms appear to be those mediated by the *mdr* family of genes (Bradley et al., 1988). These genes code for a membrane glycoprotein that is associated with an enhanced capacity to efflux certain categories of antineoplastic agents out of the cell.

Classical theories of drug resistance would predict for significant numbers of drug resistant cells in advanced tumors, but the issue as to whether very small micrometastatic burdens could also be expected to contain significant numbers of resistant cells is more problematic. If tumor heterogeneity with respect to degrees of drug resistance is still the principal reason for treatment failure, even in the adjuvant setting, then this dictates particular avenues of drug treatment strategy. However, if there are other processes involved which can be implicated in the processes of chemotherapy failure then it is likely that different approaches will need to be devised to circumvent them.

It has been postulated that if there are appreciable numbers of tumor cells residing in a portion of the cell cycle where they are less sensitive to chemotherapy (i.e. in a nondividing state) then these populations of cells might escape chemotherapeutic killing and be the basis for subsequent relapse following adjuvant chemotherapy (Mauer et al, 1977). Such kinetic factors in treatment failure would dictate different drug treatment strategies as part of the program of adjuvant chemotherapy and perhaps indicate that some types of antineoplastic agents should be used in preference to others.

EVIDENCE IMPLICATING KINETIC PROCESSES IN DETERMINING DRUG SENSITIVITY

The important studies by Bruce et al (1966) were among the first to identify kinetic factors as having a major impact on the overall drug sensitivity of both normal and neoplastic cell populations. Using the spleen colony assay for measuring both hematopoietic and tumor stem cell capacity, these workers were able to show that most chemotherapeutic agents had a significant preferential toxicity for lymphoma cells as compared to normal hematopoietic precursor cells. They speculated that much of this difference could be accounted for by the fact that a high proportion of normal colony-forming cells in the bone marrow are in a nondividing physiological G-0 state, whereas nearly all of the lymphoma cells are in the division cycle. Confirmation for this hypothesis was obtained when animals were pretreated with low doses of chemotherapeutic agents sufficient to cause extensive marrow regeneration (that is, nearly all of the resting cells in the marrow were called into the growth cycle). Under these conditions, there appeared to be little if any differential toxicity for normal versus malignant cells, with both cell types exhibiting the same degree of sensitivity to chemotherapeutic agents (Valeriote and Bruce, 1967).

In Bruce's experimental system, it is important to remember that it is the normal cells that entered in and out of a nondividing state, it appeared that with the lymphoma cells, on the other hand, that virtually the entire population was continuously in the generation cycle and under the experimental conditions employed by Bruce no evidence for a nondividing state for the tumor state was identified. However experimental systems in which it can be demonstrated that tumor cells do cease active division can be seen both in confluent cell cultures as well as tumor microspheroids (Sutherland and Durand, 1976; Durand, 1976).

In the former circumstance, it is easy to demonstrate that following initial subculturing into appropriate media that most tumor cell lines will grow almost exponentially with time until they reach some saturation density of cell number with respect to what the tissue culture medium can support. At this juncture, no further increase in cell number is observed and most cells that are actively dividing come to rest at some point in the cell cycle. Flow cytometric analysis of such cell cultures show that the characteristic distribution of cells throughout the different phases of the cell cycle are retained, though there is a tendency for reduction in the fraction of cells that are in the S-phase (Shackney and Ritch, 1982). However, the picture is consistent with cells coming to rest randomly throughout the cell cycle as opposed to entering into a physiological G-0 state. In this latter circumstance, one would expect to see a characteristic single Gaussian density of cells at the 2n value rather than the 2n and 4n peaks that are seen in most stationary phase cultures.

In cell culture systems, such cells are actually in a nutritionally deprived state and can be considered to be slowly dying rather than in a physiological non-growth condition. Whether a stationary phase cultures represent an appropriate model for nondividing tumor cells is certainly at least open to question.

The tumor microspheroid grown *in vitro* may represent a better model of the behaviour of *in vivo* tumors insofar as it is possible to identify a number of differential zones in the tumor microspheroid that appear at least superficially to correspond to the growth zonal patterns that are seen in *in vivo* experimental tumors (Durand, 1976).

Typically in the tumor microspheroid the outer rim of cells are actively proliferating and as one goes further into the depth of the spheroid less cell growth is apparent and the cells at the greatest depth in the spheroid have ceased division. If the spheroid is disaggregated and the individual cell is plated out in culture, the cells in the depth of the spheroid can resume cell division indicating that they retain their viability at least for a period of time.

Studies by a number of investigators have shown that the deeper cells within the microspheroid are less susceptible to chemotherapeutic action, at least under the conditions where the architecture of the spheroid is retained. Moreover, the ability of different cytotoxic agents to induce cell killing in the inner most cells of the spheroid is highly variable with agents such as doxorubicin and etoposide having relatively little effect on the inner most cells whereas drugs such as BCNU and cisplatin are relatively effective against the inner layer of tumor cells (Durand, 1989; Durand and Goldie, 1987).

It is not entirely clear whether this represents problems of diffusion of the drug into the depth of the spheroid or whether it represents temporary relative phenotypic resistance displayed by these cells because of the physiological conditions under which they are growing in the depths of the spheroid. It is known that factors such as pH and oxygen tension are different at different levels in the spheroid and this in turn may influence the drug effect.

However, the important point to be made is that even in the microspheroid model, the inner most nondividing cells are not invulnerable to a number of types of chemotherapeutic agents. If an analogous state exists within *in vivo* tumor systems, this could result in at least temporatory relative degrees of drug resistance, but not in the development of total refractoriness to at least some classes of anticancer drug.

CLINICAL IMPLICATIONS OF TREATMENT FAILURE DUE TO KINETIC OR RESISTANCE FACTORS

We can now examine a number of scenarios involving different hypothetical states for the micrometastatic disease at the time adjuvant chemotherapy is commenced. Depending upon whether kinetic or genetic factors predominate in dictating overall treatment failure, this should ultimately be reflected in the behaviour of the tumor at the time of relapse, as well as which empiric chemotherapeutic strategies to date appear to have been most effective. Each scenario and the possible outcomes will be discussed in turn. Although these inferences may be generalizable, we will put especial emphasis on the adjuvant treatment of stage II breast cancer as the appropriate clinical model.

Treatment Due Failure Entirely to the Presence of Drug Resistant Cells Already Present in the Micrometastasis at the Time of Chemotherapy

If drug resistance is the basic problem in the great majority of instances of treatment failure in stage II breast cancer then upon relapse the tumor should be either poorly responsive or refractory to repeat applications of the chemotherapy that was used in the adjuvant chemotherapy program. Extending the duration of the chemotherapy treatment time above some certain lower limit should make little difference to eventual outcome. Since the numbers of drug resistant cells is in part a function of the total extent of metastatic burden then we should see an inverse correlation between extent of stage of initial disease and magnitude of therapeutic impact of the chemotherapy. That is, it should be apparent that patients with stage I disease are experiencing a correspondingly much larger benefit than those with stage II 10+ nodes.

We would expect the chemotherapy programs incorporating a larger number of active agents given at relatively greater dose intensity should yield, stage for stage, more effective disease free prolongation than less complex and intensive regimens.

If a component in the treatment failure situation are relatively small numbers of partially drug resistant cells then treatment regimens incorporating periodic re-induction or intensification might be expected to be superior to regimens not utilizing this approach. This is essentially because one would anticipate that intermittent higher peak concentrations of active agents may be able to deal with partially resistant cells and to eliminate them before they evolve to higher levels of drug resistance.

The Metastatic Disease Consists Essentially of Only Drug Sensitive Cells and That Evolution to Drug Resistance Does Not Occur Over the Period of Treatment

If classic genetic drug resistance does not evolve during treatment and was not present at the time treatment was commenced this would suggest essentially that the tumor cells are overall homogeneous but with a relatively lesser degree of sensitivity to chemotherapy or (which amounts to the same thing) have very minor degrees of partial drug resistance. This would imply that the log kill per treatment cycle is relatively low although there is overall a net cytoreduction between treatment cycles. It is under these circumstances that we would expect that more prolonged periods of adjuvant chemotherapy, with or without periodic re-induction, should result in improved treatment results.

A Proportion of Cells in a Non-Dividing State Analogous to the Ones Described Previously

Under these circumstances we can postulate numbers of cells that reside temporarily or for prolonged periods in a non-dividing condition e.g. G-0 , prolonged G1 or randomly at rest throughout the cell cycle.

Experimental models of these states as described above suggest diminished drug sensitivity but not complete drug resistance. Because of their relative drug resistance there are clones of tumor cells that essentially escape being killed during the program of adjuvant chemotherapy and at some later time enter their growth phase to cause eventual relapse. The relapsing tumor under these circumstances likely would be similar in its spectrum of drug sensitivity to relapsed tumor presenting de novo and it should at a minimum be possible to generate significant response rates with re-application of the adjuvant chemotherapy drugs.

Because the resistance in this situation is relative and not absolute the effectiveness of the chemotherapy regimen can be enhanced by:

1) Increasing the dose and dose intensity of the program.

2) Ensuring that agents that have a potential for killing non-dividing cells are a major component of the protocol, i.e. alkylating agents, cisplatin.

3) Periodic cycles of more intense re-induction chemotherapy may result in further treatment benefit.

Treatment Failure is Due to a Combination of All of The Above Elements, Varying From Individual Patient to Patient

This is the most complex scenario to analyze and one in which it would be difficult to predict the overall behaviour of the tumor at relapse. One could anticipate that the relapsing tumor may show a fair degree of genetic drug resistance but this would be variable from case to case and might overall be less that what would be predicted by a straight genetic drug resistance and selection phenomenon. If it is this more complex situation, however, it does appear it would probably yield to a general application of the chemotherapeutic strategies that the other less complicated scenarios dictate. That is, that one should employ more active agents, in a more dose intense fashion, and ensure that drugs that have a capacity for killing cells in the non-dividing state are an important component of the whole treatment protocol. If the overall dose intensity of the adjuvant program is sufficient then it is unlikely that very extended treatment programs and those involving periodic re-induction would confer much additional benefit.

Cells Exist in a Non-Dividing State Different From Those Described Experimentally in Which They are Totally Invulnerable to Maximum Tolerated Doses of Cytotoxic Agents

If tumor cells can enter a state of prolonged dormancy in which they essentially are completely drug resistant then all of the above mentioned strategies will ultimately be unsuccessful. Even periodic re-induction intensification is not likely to be beneficial as the odds against catching all of the dormant tumor cells as they randomly come out of cell division would appear to be very great. If this process is operative within early stage breast cancer then generating unambiguous cures with standard chemotherapeutic approaches will likely not be effective. Only techniques that are directed at the fundamental biology of the dormant condition (perhaps combined with chemotherapy) would ultimately prove to be appropriate. These strategies could involve using agents to drive the tumor cells out of the dormant state and into the division cycle or conversely "freezing" them in their dormant state indefinitely.

SUMMARY AND CONCLUSIONS

Of all of the potential scenarios described above the last is perhaps the most pessimistic or at least the one which will require the greatest advances in understanding and modulating tumor cell behaviour.

If this type of phenomenon is indeed the basis of treatment failure in stage II breast cancer then it will be difficult to demonstrate this directly by any of the techniques currently available. If consistent application of the strategies dictated by other treatment failure scenarios fail to yield progressively improving results then one might have to infer this more pessimistic situation indirectly. This carries with it the implication that ultimate and truly satisfactory control of micro-metastatic breast cancer will have to await further fundamental discoveries involving the growth parameters and properties of tumor cells during the time when they exist as small isolated colonies.

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BIOLOGY OF RESIDUAL BREAST CANCER AFTER THERAPY: A KINETIC INTERPRETATION

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The modern management of clinical breast cancer presents a two-sided paradox. On one side is the phenomenon of early-stage tumors that should by all expectations respond well to treatment but yet fail to be eradicated, so that they eventually recur. The obverse is total drug resistance in the advanced-disease setting, in which no chemotherapy affords any benefit whatsoever. Although both situations seem, on the surface, to concern cells refractory to treatment, they are really quite distinct. The first focuses on the observation that most observable breast cancers (stages III and IV) do shrink in response to chemotherapy. It refers to the presumed shrinkage of the microscopic volumes of disease left after surgery for stages I and II disease and raises the question of why such anticancer effect does not always translate to cure. The second paradox recognizes that some advanced tumors do not regress at all even when they are exposed to chemotherapy for the first time, and that all tumors that do respond initially fail to do so eventually. The latter event is curious because the same agents which totally fail to kill any tumor cells still reproducibly kill enough normal cells to predictably cause toxic side-effects. Such refractory behavior on the part of advanced tumors, difficult to explain purely on kinetic grounds, may be an important clue as to why we fail to eliminate most tiny "sensitive" breast cancers.

Underlying the paradox of early-stage disease are several key clinical observations. Combination chemotherapy makes many advanced breast cancers regress.[1] The best studied combination is CMF (cyclophosphamide, methotrexate, 5-fluorouracil), with or without vincristine and prednisone, originally described by Cooper.[2] The use of this combination causes 34% to 68% of advanced breast cancers to shrink, and 3% to 19% to achieve complete remission with (temporarily) no evidence of disease. Such complete or partial remissions typically last for a median of 6 to 11 months.[3 4 5 6 7 8]

CMF was one of the first drug combinations to be used in the treatment of early-stage breast cancer.[9] In stages I and II the primary tumor in the breast, and metastases to axillary lymph nodes, if any, are eliminated by surgery or the combination of surgery and radiotherapy. By definition, our best diagnostic tests can detect no evidence of residual cancer. However, long-term studies of patients treated by radical mastectomy without systemic chemotherapy indicate that only about thirty percent of patients are cured by local treatment alone.[10 11] Hence, seventy percent of the population have micrometastases that will, if left untreated, grow within the natural life spans of the patients to clinical size. CMF clearly inhibits the growth of some of these tumors. When applied against early-stage disease CMF is seen to improve disease-free and overall survival, even when analyzed by conservative overview methods.[12] In the controlled trial from Milan, at ten years of follow-up an actuarial 31.4% of patients receiving no post-operative adjuvant chemotherapy were free of disease, compared to 43.4% in a group of patients who received one year of CMF. Overall survival benefit was real but more modest at 47.3% vs. 55.1% respectively.[13]

CMF, therefore, has demonstrable activity, but should we expect more from these drugs in the adjuvant setting? Two dominant ideas in the world of theoretical oncology would suggest that the answer is yes. The first idea is the original Skipper-Schabel or log-kill model of tumor growth and regression.[14] The development of this theory was a turning point in modern oncology, summarizing mountains of experimental data and allowing for the incorporation of concepts born in the laboratory into practical clinical protocols. Skipper's colleagues Simpson-Herren and Lloyd observed that leukemia L1210 in BDF_1 or DBA mice grows exponentially from implantation (of up to 10^7 cells) to the death of the mouse (at about 10^9 cells).[15] The doubling time of this tumor reflects a growth fraction (the fraction of cells actually undergoing mitosis at any one point in time) of close to ninety percent and a cell cycle time of twelve to thirteen hours. Within experimental tolerances the growth fraction and cycle time vary only slightly as the tumor grows, producing an almost constant doubling time, independent of the size of the cancer. Assuming that the doubling time T_d is constant, the time T_1 required for the tumor N to reach any specific size N_s is dependent only on the size of the tumor N_1 at the start of the observation period.

$$T_1 = T_d \cdot \ln(N_s/N_1) / \ln(2)$$

Hence, if a therapy kills some tumor cells, leaving a new starting size N_2, it will take longer for that tumor to regrow to the lethal tumor size than in the unperturbed situation. This allows for the determination of the average number of cells killed by a therapy as a function of the relative survival times of treated vs. control animals.

$$(N_1-N_2)/N_1 = 1-\exp[\ \ln(2) \cdot (T_1 - T_2) / T_d\ ]$$

Skipper used this method to learn that if a certain therapy killed x out of y cells, the same therapy applied to a tumor of size z would be expected to kill (x/y)z cells. That is, the fraction of cells killed would always be x/y regardless of the size of the malignant population. For most drugs the ratio x/y increases with increasing dose. In addition, if two or more drugs are used, each will kill its expected fraction of cells: Say drug 1 kills x_1 out of y cells, and drug 2 kills x_2 out of y cells, then the simultaneous use of drugs 1 and 2 against a tumor of size z will kill $(x_1/y)(x_2/y)z$ cells. Hence, if several drugs at adequate doses are applied against a tumor of sufficiently small size, the number of cells left after treatment may be smaller than one, which is the definition of cure. The components of CMF are all active against advanced breast cancer (large z), and hence, by this model, should be equally efficacious against the microscopic disease (small z) found in the adjuvant situation. Cure would not be an unreasonable expectation in this setting.

This optimistic view is amplified by the second dominant idea, that small volume tumors contain a higher percentage of actively dividing cells than their larger counterparts. This idea is indeed based on the detailed analysis of experimental animal tumors.[16] [17] Since most chemotherapeutic agents kill dividing cells preferentially, the fraction of small z cells killed may actually be even greater than the fraction of large z cells killed. That is, if the log-kill model is in error, the error should be in the direction of underestimating the impact of therapy against small volume tumors: If $z_1 > z_2$, and drug-schedule 1 kills x out of z_1 cells, then the number of cells killed when the same therapy is applied to z_2 should be greater than $(x/z_1)z_2$, rather than equal to $(x/z_1)z_2$ as previously postulated. Hence, it should be even more likely that CMF should have a major cell-kill impact on the z_2 cells in the adjuvant setting, considering that the z_1 cells seen in advanced disease are several orders of magnitude more numerous.

Why, then, is the curative influence of adjuvant CMF so small? The first and most obvious possibility is that some cells in z_2 are biochemically resistant to the applied dose-levels of cyclophosphamide and methotrexate and 5-fluorouracil. Skipper's current log-kill model incorporates this concept, hypothesizing that incurability is the consequence of the presence of permanently resistant cells able to overgrow the biochemically sensitive cells that regress in response to treatment. Volume expansion, according to Skipper, occurs when the ratio of resistant cells to sensitive cells exceeds one.[18] Cure can only be accomplished when therapy is initiated at a tumor size so small that no resistant cells are as yet present. This latter statement assumes that very small tumors (perhaps just a few cells in number, perhaps very close to the point of true carcinogenesis) are uniformly biochemically sensitive, with resistance emerging as an acquired trait later in the tumor's growth history. Accepting this concept, the major issue confronting the clinician is when in the time-course of growth does resistance develop, and how can tumors be diagnosed early enough to be able to start treatment when the tumor is still curable.[19]

As an aside, the problem of breast cancer metastases may be a comparable situation. Based on the work of Fidler and colleagues, it in now regarded as likely that metastatic ability arises by random somatic mutations in wild-type nonmetastatogenic precursor cells.[20] If primary breast cancers are mistreated by being allowed to grow unperturbed in the breast, as was common practice in the nineteenth century, all will become metastatic.[21] The use of radical mastectomy interferes with this progression, but seventy percent of patients will have already developed metastatic cells by the time of this intervention. In contrast if patients are treated by lumpectomy without radiotherapy, the local relapse rate is significant. Yet such patients do not have a higher metastatic rate (measured by the survival rate) than patients treated adequately de novo by lumpectomy plus radiotherapy.[22] Hence, tumor can remain in a breast, grow in the breast, and yet not develop and release metastatic cells at a high rate. That is, seventy percent of patients develop mutations before diagnosis, but the remaining thirty percent may not develop mutations rapidly even if the tumor is allowed to grow for a period. The question of when in the life-course of a tumor do mutations toward metastatic potential occur, and at what rate, is an issue highly relevant to planning optimal management.

Another biological situation related conceptually to the emergence of drug resistance is the development of resistance to viruses in bacteria. In 1943 Luria and Delbruck published an analysis of variance of a data set in which the number of virus-resistant bacteria were determined in repeated samples of each of different cultures of the same precursor bacterium.[23] The experiment was conducted so that each test population of bacteria was evaluated at the same time after exposure to the bacteriophage. If resistance to virus developed as a random function after exposure, the variance within samples and among cultures should have been the same. However, the variance within samples was much smaller than that among cultures. This was regarded as signifying that the different cultures had developed virus resistance at random different times in their growth histories prior to bacteriophage exposure, so that they already had different proportions of virus-sensitive and virus-resistant bacteria when they were first exposed to virus. These authors presented a mathematical treatment of these data, concerned with the exponential growth pattern of bacteria in culture, and the rate of random somatic mutation.

By 1952 Law had extended this method of analysis to the problem of antimetabolite resistance in L1210 cells.[24] He found that the number of resistant cells varied more among sublines than within samples of the same subline, even though all tumors were evaluated at the same time after exposure to the folic acid antagonist. From this result he concluded that, similar to virus resistance in bacteria, antifol resistance was a trait acquired randomly at different times in the pre-treatment growth history of a tumor. If a cell mutates early in the growth history of an exponential tumor, it has a long time to grow to constitute a significant percentage of all cells present. A tumor whose first mutation toward resistance is late in its history (closer to the point of analysis) has a smaller percentage of resistant cells.

The subsequent decades saw the discovery of myriad biochemical mechanisms of drug resistance.[25] These were recognized quite early to be important in clinical cancer treatment.[26] Law's kinetic observations, however, were not influential in the design of anticancer therapy until 1979 when Goldie and Coldman published their mathematical treatment of the Luria-Delbruck model.[27] The model may be restated as follows: A cell mutates with probability α at each mitosis. The probability of the cell not mutating in one mitosis is therefore $(1-\alpha)$. If a cell undergoes x mitoses, the probability P_0 of no mutations occurring is $(1-\alpha)^x$. Since $\log_e(1-\alpha)^x$ is approximately $-\alpha x$ for small α, then P_0 is approximately $\exp(-\alpha x)$. Assuming that each mitosis produces two viable cells, and that there is no cell loss, a chain of N-1 mitoses starting from one cell will yield N cells. Hence, $P_0(N)$ is approximately $\exp(-\alpha(N-1))$. Examining $P_0(N)$ as a function of $\log_{10}(N)$, Goldie and Coldman observed that the probability of finding no mutants changed rapidly over a two-log change from say 10^5 cells ($P_0=.905$ at an α of 10^{-6}) to 10^7 cells ($P_0=.000045$). It is of note that 10^7 cells is a tumor volume of only .1 cubic centimeter at a packing ratio of tumor to benign stromal (or other) host cells of 1:10. The model was later expanded to include multiple sublines and cell loss,[28] but the three fundamental conclusions remained intact: (1) Failure to cure is a consequence of the presence of drug-resistant cells that arose by random somatic mutation before the initiation of treatment or during treatment; (2) Since drug resistance can emerge rapidly, early institution of therapy is essential; (3) Applying therapy to make N as small as possible, as rapidly as possible, is important to maximize P_0 in the residual cells.

Clinically, the first expectation of the Goldie-Coldman model is that tumors relapsing after adjuvant chemotherapy should be comprised mostly of drug-resistant cells. The second expectation is that treatment instituted as early as possible after surgery should be more effective than delayed treatment. The third is that as many effective drugs as possible should be applied as soon as possible. This last statement merits further exposition: Imagine that N is the sum of three populations $N_o + N_a + N_b$. N_o is sensitive both to drugs A and B, N_a is sensitive only to drug A, having mutated to resistance to drug B, and N_b is likewise sensitive only to drug B. If only drug A is used, the N_o and N_a cells will respond, but the N_b cells will grow and rapidly mutate to resistance to B and other drugs as well. Hence, the model concludes, both A and B should be applied. This recommendation is intuitive and conforms to the already established principles of combination chemotherapy.[29] It differs, however, from these classic principles in that it emphasizes the possibility that resistance may emerge during treatment, as contrasted with the likelihood that resistance is already present at the start of treatment. Furthermore, the Goldie-Coldman model asserts that if A and B cannot be used simultaneously at good therapeutic levels (because of overlapping toxicity, competitive interference, etc.) they should be used in alternating sequence ABAB....The authors note that this recommendation is highly dependent on the assumption that N_a cells are as sensitive to A as N_b cells are to B, that α is constant in both sublines, that the growth kinetics of N_a and N_b are equivalent, and that A and B are somewhat (not necessarily completely) noncross-resistant.[30]

Several clinical trials in breast cancer have examined these issues; Some are still in progress. The results to date do not support the above expectations. In 1980 the Cancer and Leukemia Group B (CALGB) initiated a study of the treatment of patients with advanced breast cancer, randomizing them to receive cyclophosphamide, adriamycin, and 5-fluorouracil (CAF) with or without the simultaneous use of the antiestrogen tamoxifen. There were 375 analyzable patients who had not received prior adjuvant chemotherapy, and 46 who had. The complete plus partial response rates in both groups were comparable: 59% in those who had not received prior chemotherapy, 50% in those who had relapsed in spite of having received adjuvant chemotherapy (p=.22).[31] There were no significant differences in response between the two groups within subsets by therapy received, sites of disease, time from first diagnosis, or performance status. Response duration (p=.47) and overall survival (p=.59) were also not different. If the patients who had received prior chemotherapy had relapsed because of the presence of biochemically resistant cells, their disease should not have retained the capacity to respond to subsequent chemotherapy. Hence, it is reasonable to question the assumption that the failure to cure patients in the adjuvant setting is entirely due to cellular drug resistance.

Additionally, the necessity for immediate institution of chemotherapy post-operatively is disputed by data from the Ludwig (International) Study Group. Their Trial V randomized 715 premenopausal and 514 postmenopausal node-positive patients to either (1) one month of immediate CMF (plus leucovorin to reduce toxicity) to start within 36 hours of surgery, (2) conventional CMF (plus prednisone and tamoxifen) to start 25-32 days after surgery and to continue for six months, or (3) immediate chemotherapy followed by conventional chemotherapy. At a median of 42 months of follow-up the immediate CMF arm is inferior to the other two but, in terms of disease-free and overall survival, there is no difference as yet between the conventional (delayed) CMF and the immediate plus conventional CMF.[32] If drug resistance emerges rapidly during unperturbed tumor growth, one would imagine that the 25-32 days of no treatment should bias that arm toward inferior results, which is not observed in this study.

Regarding the expected superiority of alternating sequences of chemotherapy, the CALGB randomized volunteer patients with advanced disease to be treated with either (1) CAF, (2) vinblastine, adriamycin, thiotepa, halotestin (VATH), or (3) CMF with vincristine and prednisone (CMFVP) alternating with VATH.[33] The VATH regimen was chosen because it is an effective regimen for patients relapsing from or failing to respond to CMF, and therefore demonstrates noncross-resistance to that combination.[34] Of the 497 patients randomized, 464 were candidates for a preliminary analysis. Contrary to expectations, the alternating arm was not superior to the other two. Complete plus partial response were 52% for CAF, 58% for VATH, and 52% for VATH/CMFVP. Complete remission occurred in 11-12% of patients in each of the three arms, and there were no differences in survival.

The Goldie-Coldman model is based on solid biological principles and is mathematically valid. It has rekindled an interest in the kinetics of development of drug resistance, and may well pertain to certain biological phenomena. It is, however, acknowledged to be dependent upon certain assumptions, and these assumptions may not apply to human breast cancer. Hence, it cannot be relied upon to explain our failure to cure more breast cancer in the adjuvant setting.

Returning to the Skipper-Schabel model, it is clear that while biochemical resistance is a convenient explanation for therapy failure, it is not the only reasonable possibility. In the model the achievement of cure demands that the residual tumor size after a three-drug therapy, given by $z-z(x_1/y)(x_2/y)(x_3/y)$, be less than one. For this to be accomplished the fractional kill (x_i/y) for each drug application must be substantial.

For this reason, the log-kill concept implies that failure to cure may be the consequence of insufficient dose-intensity of one or more of the component agents. A tumor may relapse because some of its cells, actually sensitive to the agents applied, are not exposed to enough drug. This is conceptually similar to a bacterial infection relapsing because of an insufficient dose or duration of treatment with an antibiotic to which the micro-organisms are sensitive. Were the case of post-operative adjuvant chemotherapy of breast cancer comparable to this latter clinical situation, it would explain the sensitivity to chemotherapy (in terms of response rate) of breast cancers relapsing in spite of having received the same drugs previously. That is, the relapsing breast cancers retain the ability to respond to chemotherapy because they never lost the ability to respond during primary (adjuvant) treatment, but were merely undertreated. This explanation would be consistent with Hryniuk's retrospective series, in which protocols that delivered more chemotherapy seemed to accomplish longer durations of disease-free status.[35] (It is recognized that such an important correlation cannot be determined from retrospective examinations, but requires a prospective, randomized clinical trial. A study with this goal is currently in progress in the CALGB) It is certainly true that in L1210 insufficient treatment can be the cause of failure to cure. However, L1210 is most frequently incurable *not* on kinetic grounds, but because of drug resistance. The usefulness of the log-kill model in understanding breast cancer, therefore, may depend upon a more detailed appraisal of the validity of its assumptions in the clinical context. Why is drug resistance an adequate explanation for treatment failure in L1210, but not in clinical breast cancer?

In addressing this question we must recognize that the log-kill model in all of its various versions was formulated from, and is expressed in terms of, exponential growth. Does human breast cancer grow exponentially? There are several lines of evidence suggesting that the answer is no. In the nineteenth century, before Halsted's popularization of the radical mastectomy, a commonly accepted treatment for breast cancer was no surgery, no effective treatment at all. In 1962 Bloom published a survival curve for 250 such women, carefully observed in the Middlesex Hospital in London.[21] Speer, Retsky, and colleagues used this curve and other data to fit a model in which tumors grow in randomly increasing steps of Gompertzian plateaus.[36] However, these data can be fit more parsimoniously, and with greater accuracy, by a family of simple Gompertzian curves.[37] Although a family of exponential curves can be fit to these data, a comprehensive model based on exponential growth cannot account for both disease-free-survival and survival results. The Gompertzian curve is the simplest curve that works. The Gompertzian model also fits data on the growth of mammographic shadows of primary breast cancers[38] and disease-free-survival following mastectomy.[39] Adding to the appeal of the Gompertzian curve is the observation that the growth of most experimental animal tumors are described by this function.[40]

The Gompertzian equation is given by

$$N(t) = N(0)\ \exp\{\ \ln[\frac{N(\infty)}{N(0)}]\ [1-\exp(-bt)]\}$$

N(0) is the tumor size at time t=0. N(∞) is the plateau size that is approached at t approaches infinity. The family of curves that fit clinical data is generated when the parameter b is allowed to assume a log-normal distribution.

What is the behavior of a Gompertzian tumor in response to effective chemotherapy? Analysis of experimental data[41] and (conceptually) clinical data[42] has suggested that the rate of tumor regression in response to treatment is positively related to the dose of therapy administered and the growth rate of the unperturbed tumor just prior to treatment. This can be expressed as the differential equation

$$dN(t)/dt = b \cdot N(t) \cdot \{\ln[\frac{N(\infty)}{N(t)}]\} \cdot \{1-D(t)\}$$

where D(t) is zero prior to treatment and a non-negative function of t after treatment. This solves to

$$N(t) = N(0)\ \exp\{\ \ln[\frac{N(\infty)}{N(0)}]\ [1-\exp(bt+b\int_0^t D(u)du)]\ \}$$

The meaning of this equation is that a slow-growing tumor (small b) will tend to regress more slowly in response to a given therapy (same D(t)) than a faster-growing tumor (larger b). Also, the rate at which any individual tumor will regress in response to treatment will be maximum when the product $N(t) \cdot \{\ln(N(\infty)/N(t)\}$ is maximum, which occurs at the size $N(\infty) \cdot \exp(-1)$. If a treatment is started when a tumor is below this size but visible (stage III or IV), the rate of regression will be faster initially than when the tumor shrinks below the level of clinical appreciation. As a consequence therapy that causes regression of a large (stage III or IV) breast cancer will not necessarily be sufficient to eradicate that tumor. The equation also states that very small tumors, as found in the adjuvant setting, will regress more slowly that some larger tumors found in the advanced disease setting. (A tumor so large that it exceeds $N(\infty) \cdot \exp(-1)$ in size will also regress more slowly.) As a consequence a therapy that is known to be able to cause regression of a large tumor will not necessarily be able to cure a smaller (stage I or II) breast cancer. Also, a therapy that can prolong the disease-free-interval in the adjuvant setting will not necessarily be able to cure the tumor, within any reasonable treatment duration.[43]

In terms of cell kill, the above equation states that the fraction of cells left after an effective treatment is related both to the size of the tumor at the time of treatment and the dose of therapy administered. If treatment is given at time t_{rx}, then at some point after treatment, say time t_m, the tumor size will be at a minimum size $N(t_m)$. Both $N(t_{rx})$ and the ratio $N(t_m)/N(t_{rx})$ will decrease monotonically as t_{rx} decreases. That is, if therapy is started early, the fractional kill will indeed be greater. However, the time t_g required for $N(t_m)$ to regrow to a size $N(t_m+t_g)=N(t_{rx})$ will be independent of $N(t_{rx})$. That is, more fractional kill results in more fractional growth, so that the eventual size of the tumor (as the influence of therapy wanes) will be the same in all cases. Hence, the time required for N(t) to equal a lethal size will be the same whether the treatment is given as a moderate, small, or very small tumor size. This may explain why overall survival is less affected by adjuvant chemotherapy than disease-free survival. It may also explain, by an extension of the argument, why the survival duration of patients with stage IV disease has remained fairly stable in recent decades in spite of more aggressive approaches to management.[44 45 46]

Since, according to the Gompertzian model, just moving therapy earlier and earlier in the growth history of the tumor cannot be expected to lead to cure in all cases, the only prospect for advance in the treatment of early-stage breast cancer is found in more intensive therapy at a very small $N(t_{rx})$. Since $N(t_m)/N(t_{rx})$ does indeed decrease as $N(t_{rx})$ decreases, early initiation of treatment is an important part of the solution, but it is not the entire solution. A more aggressive chemotherapeutic approach (higher doses, more frequently administered) might increase the definite integral of $D(t)$, and thereby might cause more rapid regression.[47] A rate of regression rapid enough to drive $N(t_m)$ below the "cure volume" (one cell or several, depending on immunological or other host factors) is the only chance for cure.

It is important to note that the above conclusions are valid even in the absence of biochemically insensitive cells. They are not, however, invalidated by the presence of such cells. Indeed, they are even more appropriate if resistant cells are present. In this context, the truly resistant cells can be defined as those for which $D(t)=0$ for all dose levels of the drugs employed. In that case, the only possible avenue for cure is the use of intense therapy using new, active agents at a very small $N(t_{rx})$. By this approach $D(t)>0$ for $t>t_{rx}$, and the cure volume could, theoretically, be achieved. In contrast with the use of a strict alternation of two non-crossresistant combinations, this technique applies one combination until $N(t_m)$ is achieved, crossing over to another drug or combination at that critical time.

The use of cross-over intensification has been successful in the laboratory. Skipper has concluded that the best (in fact, the only) way to cure 10^8 L1210 cells is the use of an induction with cytosine arabinoside plus 6-thioguanine for two or three courses, followed by a single simultaneous dose of cyclophosphamide and BCNU.[48] Griswold and colleagues, in a series of experiments specifically addressing the problem of tumor cell heterogeneity in drug sensitivity, exposed BDF_1 mice bearing the M5076 tumor to various schedules of Methyl-CCNU and 1-phenylalanine mustard (1-PAM).[49] Five weekly administrations of 1-PAM induced no complete remissions (CR) and caused a median percent improvement of life span of only 51%. MeCCNU on a similar schedule caused 40% CR and median % ILS of 71%. However, four weekly doses of MeCCNU followed by a single dose of 1-PAM gave a CR rate of 80%, with a median % ILS of 138%. A single dose of a drug which by itself could induce no complete remissions was able to double the CR rate and almost double the median % ILS. The mechanism for this effect is presumably that the few cells left after MeCCNU induction are 1-PAM sensitive. However, in the untreated situation, most cells are MeCCNU sensitive, 1-PAM resistant. These data support the notion that cross-over therapy can be beneficial if appropriate drugs, doses, and timing are employed.

In 1980 the CALGB initiated a study, based on these concepts, of the adjuvant chemotherapy of node-positive stage II breast cancer patients post-mastectomy. Patients were randomized to receive eight months of CMFVP by one of two different schedules. Those still in complete remission at that time were randomized to either continue their CMFVP schedule or cross over to six months of VATH in a less-than-dose-intense (for safety and feasibility) schedule. The two versions of CMFVP delivered about the same dose-levels of drug (on a relative scale[35]) and gave the same clinical results.[50] Hence these data were pooled to yield a two-arm comparison of fourteen months of CMFVP vs. eight months of CMFVP followed by six months of VATH. In a preliminary analysis of 897 patients, those receiving the cross-over therapy had a significantly improved disease-free survival (p=.01).[51] Subset analysis suggested that patients with four or more involved axillary lymph nodes, and those beyond menopause, received particular benefit. This trial will, of course, be followed carefully for further evidence of effect. It cannot be concluded that VATH following CMFVP is superior to regimens not tested, such as CMFVP alternating with VATH, or even VATH alone. But these results do indicate that VATH given at a significant time period after mastectomy does have a real biological effect. That is, resistance to VATH does not arise quickly in residual cells resistant to CMFVP. This is further evidence against the appropriateness of the Goldie-Coldman model in post-operative clinical breast cancer, and lends support to the possibility that the above Gompertzian analysis may be profitably applied in the future design of innovative chemotherapy schedules.

It is of note that the VATH in the CALGB adjuvant protocol was not given in a dose-intensive fashion, and therefore does not satisfy the full requirements of the model, and does not duplicate Skipper's L1210 experiment. It is possible that a more aggressive use of agents in the cross-over, perhaps using adriamycin in the induction (to avoid evolution in expression of the multidrug-resistance gene[52]) followed by intensive-dose alkylating agents, would lead to truly important clinical results. This forms the theoretical basis for a current CALGB pilot study treating stage II patients with ten or more involved axillary lymph nodes with cyclophosphamide, adriamycin, and 5-fluorouracil (as induction), and high-dose Cisplatin, cyclophosphamide, and BCNU, with autologous bone marrow rescue (as intensification). Such an approach shows promise in breast cancer and other diseases.[53]

While we wait for further laboratory and clinical data to accumulate, it may be of value to speculate about _why_ rate-of-regression may be positively related to rate-of-growth. At first blush it may seem reasonable to hypothesize that tumors regress most rapidly when more of their cells are undergoing mitosis, because these are the very cells at risk for cytotoxic damage from the drugs. This is a possible explanation for the phenomenon, but it is not logical _prima facie_. If about five percent of breast cancer cells are cycling at any one time in an average tumor,[54] then a maximum of five percent of the tumor mass should be killed by a single-shot therapy. To get a one-log kill (90% regression) would require forty-five such shots! This would mean that with each cell death, another cell must be immediately recruited into mitosis, and then must be immediately killed by the presence of the active drug, for forty-five cycles. Considering the long length of cell-cycle times in mammalian neoplasia, the slowness of recruitment, and the short half-life pharmacology of many active agents, such a process may not be possible. In addition, histological analysis of regressing tumors does not always confirm the high degree of necrosis that would be expected with rapid cell lysis. Is there another possible explanation for the chemotherapy effect?

It is now clear that breast cancer growth is modulated by endogenous growth factors secreted by a subset of tumor cells in an individual cancer.[55] It is reasonable to expect that a tumor with a higher specific activity of growth promoting factors (a higher percentage of growth-factor-producing cells, or higher growth factor production per cell, or higher receptor activity in producing or responding cells, etc.) will grow faster than a tumor not so well endowed. If the primary target of chemotherapy is growth-factor-producing cells (either killing them, or otherwise impairing their productivity, or stimulating the production of growth inhibitory factors), then growth-factor-responsive cells will be deprived or suppressed, and regress passively. Tumors growing most rapidly because of higher specific growth factor activity would therefore regress most rapidly when the growth factors are perturbed. Secretion of inhibitory growth factors is, after all, a possible mechanism for antiestrogen effect in a hormone-responsive breast cancer cell line.[56] Also, growth factor perturbation may very well be part of the mechanism of chemotherapy-induced bone marrow suppression. In evidence is the observation that supplying exogenous leukocyte growth factors such as granulocyte colony stimulating factor partially blunts and reverses with rapidity the leukopenia of chemotherapy.[57] This argument raises the question: Does chemotherapy exerts an activity similar to antiestrogens in clinical breast cancer?

In the laboratory we can easily evaluate individual tumors or individual collections of cells. In the clinic, however, we are usually restricted to analyzing cohorts of patients, and then rarely tumor growth rates, but most commonly indirect measurements such as time to relapse or survival time. There have been notable attempts to use time-to-event curves to produce hypotheses about biological events. For example, Harris and Hellman noted that survival after mastectomy appears to be approximately biexponential, suggesting that two distinct populations of patients, each with their own exponential force of mortality, may be mixed in the usual clinical setting.[58] Skipper used the breakpoint in survival curves (the point at which the survival curve deviates sharply from exponentiality) to estimate cell repopulation kinetics in mice bearing various experimental neoplasms, and applied this method to clinical breast cancer data.[59] Holland presented a similar analysis of other clinical data.[60] Henderson examined curves with and without adjuvant CMF, estimating an average benefit by taking the difference between the times required to reach the sixty-percent actuarial survival probability.[61]

By the Gompertzian equation

$$\ln(T) = \ln\{-\ln[\ 1 - \frac{\ln(\frac{N(T)}{N(0)})}{\ln(\frac{N(\infty)}{N(0)})}\ ]\} - \ln(b)$$

If $N(0)$ is below clinical appreciation and if we let $N(T)$ represent the detectable tumor size, T is the disease-free survival time. If b is log-normally distributed, T should be log-normally distributed, given constant starting size $N(0)$ and plateau size $N(\infty)$. Estimating the probability distribution of T from actuarial curves of long-term adjuvant tamoxifen data[62] and CMF data[13] does indeed confirm a normal distribution of $\ln(T)$. In both cases, therapy shifts the mean $\ln(T)$ considerably to the right, with reduced variance. This could signify that the probability distribution of b is altered by treatment (tumor regrowth is slowed), or that $N(0)$ is reduced more in fast-growing tumors than is slow-growing ones (as predicted by the Gompertzian model), or some complex relation involving these and other effects. The interesting observation is that, despite differences in degree, the alteration in pattern of $\ln(T)$ after adjuvant CMF is similar to that associated with the impact of tamoxifen.

Curve-fitting analyses of this type, of course, are productive only to the extent that they suggest hypotheses verifiable by the scientific method, preferably in the laboratory. Thus it may be reasonable to look for chemotherapy effect in the perturbation of growth factor loops in biological models. It would be interesting to screen for antineoplastic effect in the laboratory in this way. The confirmation of such an hypothesis would indeed go a long way toward illuminating the double-sided paradox that introduced this exposition. By this hypothesis, the mechanism for response in all stages of clinical breast cancer is the inhibition of subpopulations producing autocrine and paracrine growth promoting factors. Let the total tumor cell population N be comprised of subpopulations $N_r + N_a + N_b + N_c$. N_r cells do not produce growth factors but are responsive to the growth factors produced by cells N_a, N_b, and N_c. Drug A eliminates the growth promoting factor production of N_a cells, drug B effects N_b cells, and drug C effects N_c cells. If only drug A is applied, there is a slight reduction in growth promoting activity, and N_r cells regress to some degree, but recover as the specific activities of N_b and N_c cells increase. If therapy consists of adequate doses of A and B, only N_c cells are left to produce positive growth factors, and the N_r cells regress considerably more than in the single-agent situation, but are not eradicated. Hence, effective combination chemotherapy can significantly perturb N, but not lead to cure. In this hypothetical situation, if A and B and C are given in adequate dose levels, all growth promoting factors can be removed, and N_r will regress to a small size, and not be able to recover. This hypothesis, therefore, does not preclude the possibility of cure with conventional therapy, but does recognize that the probability of cure is lower than would be anticipated by conventional concepts. Regarding the total unresponsiveness of some advanced cancers de novo, this could because of the presence of a large population of growth factor-producing, drug-resistant cells or, as tenable, the existence of many N_{nr} cells, not responsive to growth factors because they are growth factor-autonomous. Since N_{nr} cells do not require growth factors to grow, depriving them of growth factors would not be expected to alter their growth pattern. Tumors that respond to chemotherapy but later relapse may do so because of the presence of a small population of drug-resistant growth factor-producing cells, or a small component of N_{nr} cells. Hence the difference between responsive (but incurable) and totally unresponsive tumors is merely a difference in quantity, not quality, of cellular subspecies.

The difficulties that we encounter in eradicating "sensitive" breast cancers in the adjuvant setting, and obtaining and maintaining tumor responses in the stage IV situation, are two of the most important clinical problems in modern oncology. By log-kill theory -- well established in the mouse laboratory -- our therapies should do better. While it is convenient to hypothesize that biochemical drug resistance is the sole explanation for treatment failure, the most popular current mathematical formulation fails to predict consequential clinical results. Part of the reason may be that exponential growth and regression -- so useful in the laboratory -- may be inapplicable to human disease. The pattern of regression of a Gompertzian tumor, which is, in essence, merely an extension of log-kill principles to a clinically relevant growth pattern, can explain some clinical observations, and may be able to guide the development of better treatments. The growth factor hypothesis is but one of many possible explanations for the presumed validity of

Gompertzian regression. Yet, the advantage of phenomenological modeling is that while it can suggest hypotheses such as these, it is not dependent upon a biological understanding to be helpful empirically. It is anticipated that further investigation at the bedside, the computer workstation, and the laboratory bench may help unravel these mysteries, for the sake of better science, and of better clinical applications.

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SURGERY, KINETICS AND BIOLOGICAL CONSIDERATIONS IN PLANNING ADJUVANT THERAPY PROTOCOLS

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INTRODUCTION

The International Breast Cancer Study Group (IBCSG, formerly Ludwig) has recently published results from a clinical trial evaluating combination cytotoxic chemotherapy given immediately after surgery (Ludwig Breast Cancer Study Group 1988, a and b, and 1989). This trial provides important data to generate new relevant questions for clinical trials and also for understanding mechanisms whereby surgical intervention might influence the kinetics of micro residual metastases. This paper briefly considers the potential for surgery to influence metastatic disease, and reviews data from Trial V specifically to identify important new questions relevant to planning protocols - including the use of biological modulation agents, the optimal timing for their use, and the future role of their use, and the future role of surgery in treatment planning and delivery. A new concept is presented to link growth and behaviour of metastases to a primary malignant process of transformation and recruitment without the necessity for the conventional dogma of cell transfer.

I. THE EFFECTS OF SURGERY ON METASTASES.

Surgery may alter the kinetics of metastatic cell growth by causing immunosuppressions, physiological stress, or by changing the balance and control of biological modulating agents.

Surgery and Immunosuppression.

Not surprisingly surgery produces measurable deficiencies in immune function, and suppression of T cells, antibody production, and cellular immunity have all been described. The physiological stress of surgery, anaesthesia, and for example, transfusion, are probably important. The impact of these effects on micrometastases probably of the order of 10^6 cells or less is unknown. It is unlikely however to be advantageous and it is a sound reason for minimizing the extent of local treatment wherever more extensive therapy is not dictated by sound scientific data. Radiotherapy is also immunosuppressive and should not be given without a clear therapeutic objective. It is likely that more precise immunological assessment using receptor and m-RNA detection technology will allow more precise measurement of both the (deleterious) effects on the immune system of surgery, radiotherapy and cytotoxics, and also the particular defects in individual patients. This would facilitate "replacement" or modulating therapy.

Surgery is also known to influence the growth of metastases. The mechanisms of this are not known, but could involve non-specific effects of operative stress, including corticosteroid and other hormone response or a more specific effect consequent upon the particular operation and log reduction of the primary tumour.

Surgery & Modulation of Tumour Growth.

There is little direct data available to describe the behaviour of micrometastases and the influence on them of modulating factors. Residual disease may be heterogeneous in terms of steroid receptor expression; growth kinetics; resistance to cytotoxic chemotherapy; production and/or susceptibility to growth factors by autocrine or paracrine mechanisms, receptors for growth factors, oncogene control and expression as well as many other phenotype characteristics that may be subject to modulation.

The removal of a primary tumour can reduce the total cell log burden. It might also remove a source of stimulatory growth factor able to influence at least some cells in residual disease, or remove a relatively large pool of cell receptor necessary for modulating agents thus allowing increased concentration of modulating agents to be temporarily available. These potential agents and mechanisms are discussed in detail throughout this symposium.

The important feature is the potential for a sensitive and delicate balance between stimulatory and inhibitory factors acting on residual micro tumour burdens of perhaps no more than a few hundred thousand cells and the potential for anti-tumour therapies - particularly surgery and radiotherapy - to modulate this balance. For breast cancer, there is good evidence that survival is not influenced by a range of local primary treatments including radical surgery (total mastectomy and axillary dissection); lesser surgery with local primary excision, axillary dissection and radiotherapy to the residual breast tissue; and local excision and axillary dissection without radiotherapy (Fisher B et al., 1989).

It would be tragic if inadequate local treatment compromised cure by other treatment modalities or facilitated locally recurrent disease that was impossible to treat. Even when dealing with tumours diagnosed apparently earlier, as for example those seen in breast cancer screening programmes, the residual burden may well be very low after local therapy, perhaps only a few thousand distant cells, and even this burden may well be critically and equally tragically influenced by the effects of local surgery with compromise of subsequent cure.

There is indirect evidence as to the size of residual tumour deposits that might be subject to surgical modulation. It is probably about 10^6 viable cells or less. Evidence comes from the size of curable melanoma, and the cell number required to establish tumours in susceptible animal models. Melanoma is detected and diagnosed early because it is readily visible. Tumours less than 0.7 mm thick are virtually curable by surgery alone. If composed entirely of viable tumour cells this might represent up to about 800,000 cells. For tumours larger than 1 mm in diameter, cures are not always seen and presumably around 10^6 cells, metastases are established for this tumour. This could reflect the frequency of mutations necessary to generate sufficient metastatic potential or the cell number that is controllable (no metastases) by a balance of growth factors, lymphokines and immunological mechanisms.

In many experimental systems, about 10^{5-6} viable cells are required to establish tumour growth. Frequently, 10^7 or more cells leads to established tumour growth, 10^5 cells or less often fails. Again, it seems plausible that autocrine, immunological, and endocrine balances can control or eradicate up to about 1 million cells, but probably not much more than this and in many cases perhaps only very much less.

II. EARLY POST OPERATIVE SYSTEMIC THERAPY IN BREAST CANCER.

With this brief background, we can consider the recently published data from the IBCSG Trial V for populations of breast cancer patients likely to have different residual cell burdens and kinetics as well as different steroid receptor cell status. This will allow relevant questions to be identified for adjuvant protocol planning based on biology - not just tumour removal.

These trials which include both larger tumour burden node positive, and smaller tumour burden node negative patients, are identified collectively as "Trial V" and have recently been published (Ludwig Breast Cancer Study Group 1988 a and b, and 1989). For this interpretation, particular biological features of possible relevance to kinetics of residual tumour, need emphasis.

Biological Features of Trial V.

1. All patients had operable breast cancer not detectable beyond the breast and axillary lymph nodes. Although this included some larger tumours, and some patients with a large number of nodes involved, it did not include patients with overt bulky disease. Patients were identified following axillary dissection as N- (no disease found in axillary glands); N+1-3 (one, two or three lymph nodes involved); N+4-9; or N+10 (10 or more lymph nodes involved).

2. **Steroid Receptor Status of Primary Tumour.**

   Where possible, a quantitative assay was completed on a primary tumour specimen for estrogen (ER) and progesterone (PR) receptors. Patients were classified as ER+ (10 or more fmol); ER- (0-9 fmol), and unknown.

3. **Age and Menopausal Status.**

   Patients were stratified into two groups according to their established functional menopausal status : pre or peri menopausal (24-60 years, median 45 years); and, post menopausal (45-73 years, median 59 years).

4. **Treatments.** (Table 1)

(a) For N- patients, random allocation of adjuvant systemic therapy was to early post operative chemotherapy (PT) or no adjuvant therapy (control). For PT the combination used was Cyclophosphamide 400 mg/m^2 i.v.; Methotrexate 40 mg/m^2 i.v.; and Fluorouracil 600 mg/m^2 i.v.; all on days 1 and 8 following surgery, with the first dose given within 36 hours of operation. The majority of such patients were treated also with Leucovorin, 15 mg. i.v. day 2, and 15 mg. orally day 9 because of possible toxic effects from interaction between Methotrexate and nitrous oxide (Ludwig Breast Cancer Study Group, 1983). Treatments were the same for both the pre or peri menopausal group and the post menopausal group.

(b) For N+ patients there were three randomly allocated alternative systemic adjuvant treatment programmes: (i) PC, (as above); (ii) conventionally timed adjuvant systemic therapy, (CT); and (iii) PT followed by CT (Table). The CT consisted of six cycles of CMFp, the first cycle starting 25-32 days after operation. Post menopausal patients treated with CT chemotherapy also received six months treatment with Prednisone (7.5 mg. orally daily) and Tamoxifen (20 mg. orally daily). Pre menopausal patients treated with CT chemotherapy also received Prednisone 7.5 mg. orally daily but no Tamoxifen.

Table 1 : **Trial V Adjuvant Regimens**.

Node Negative Adjuvant Regimens:

Pre and Peri Menopausal

A. Early CMF x 1
B. Nil (no adjuvant therapy)

Post Menopausal

A. Early CMF x 1
B. Nil

Node Positive Adjuvant Regimens:

Pre and Peri Menopausal

A. Early CMF x 1
B. Delayed CMFp x 6 (conventionally timed)
C. Early CMF x 1 + Delayed CMFp x 6

Post Menopausal

A. Early CMF x 1
B. Delayed CMFp x 6 + Tamoxifen (T)
C. Early CMF x 1 + Delayed CMFp x 6 + T

Node Negative Results. (Table 2)

At a median follow up of 42 months the four year disease-free survival was 77% (±2) for patients receiving early perioperative chemotherapy, and 73% (±2) for those not receiving this adjuvant chemotherapy (hazard ratio 0.77; 95% confidence interval, 0.61-0.98; p=0.04). This benefit for an early single course of CMF was apparent for both the pre menopausal population as well as for the post menopausal population. Retrospective analysis by estrogen receptor content showed a greater advantage for early perioperative CMF for the population with estrogen receptor poor tumours. This very early analysis demonstrated a small survival (non-significant) advantage for the PC.

Node Positive Patients.

At 42 months median follow up there was a statistically significant difference in outcome for both pre and post menopausal populations. The Disease Free Survival (DFS) results were (% at 4 years ±SE) : premenopausal patients PC 40 ± 4; combined 63 ± 4; CT 62 ± 4. ($p<0.0001$). For post menopausal patients, the results were 40 ± 4, 55 ± 5, and 63 ± 4 respectively; $p=0.10$ (Table 3).

Subgroup Analyses and Interpretations Relevant to Kinetics.

Analyses of results by number of nodes involved and oestrogen receptor status is done retrospectively such that subgroups analyses may be subject to important but unknown bias. This must be remembered when interpretations of the data are made.

(i) Nodal Involvement & Treatment.

For both the pre and post menopausal groups (which were concurrent independent trials) there may be a consistent difference in outcome between the better prognosis, probably smaller residual burden group, N+1-3, and the much poorer prognosis, probably larger residual tumour burden group N+10.

Thus, for the N+1-3 group, for both pre and post menopausal patients and for both DFS & OS, the two treatments with longer duration therapy may be superior to the single early perioperative therapy (Table 2).

For both pre and post menopausal groups and DFS & OS the N+10 group appears to show an advantage for the conventionally timed therapy alone, with little or no difference between the early perioperative treatment and the combined arm (Table 2).

Table 2: **Four Year DFS Percentages and OS Percentages by Treatment Group and Nodal Status, at median follow-up time of 42 months.**

	Adjuvant Therapy Group (percent +SE)			
	Early Perioperative Only	Combined	Later Conventionally Timed Only	P Value *
Premenopausal				
DFS:				
All patients	40±4	63±4	62±4	<0.0001
N+1-3	53±5	77±4	76±4	<0.0001
N+10	16±6	19±8	34±10	<0.004
OS:				
All patients	66±4	73±3	78±3	0.07
N+1-3	75±5	86±3	85±4	0.12
N+10	36±10	39±9	69±10	0.02
Postmenopausal				
DFS:				
All patients	40±4	55±5	63±4	0.0001
N+1-3	51±6	60±7	70±5	0.06
N+10	24±9	15±9	36±11	0.42
OS:				
All patients	73±4	76±4	82±3	0.10
N+1-3	81±5	81±5	85±4	0.49
N+10	39±4	46±12	68±2	0.16

DFS = Disease Free Survival
OS = Overall Survival

* Calculated with use of a two-sided log-rank test for heterogeneity among treatment groups.

These differences are not constant for each group to the same degree. The fact that they are similar however in the two concurrent but independent trials makes them important and worthy of discussion. Before proceeding further it is important to first look at estrogen receptor subgroups in an attempt to distinguish between those effects possibly due to Tamoxifen (and possibly Prednisone) effect in the post menopausal trial and those possibly related to the timing, duration and impact on residual tumour effects of the cytotoxic chemotherapy.

(ii) Estrogen Receptor Status and Treatment (Table 3)

For post menopausal ER+ patients, there is no demonstrable difference between the two Tamoxifen containing arms (combined and later conventionally timed) and both seem superior to the early perioperative therapy only arm for both Disease Free and Overall Survival.

For post menopausal ER- patients there is no advantage for the combined arm over early perioperative therapy alone and both seem inferior to the later conventionally timed therapy arm alone for both DFS & OS.

For the pre menopausal ER+ patients the data are less clear. In terms of DFS, both the combined and later conventionally timed arms seem superior to the early perioperative arm alone. For OS, the delayed conventionally timed arm may be the best, but at this stage of follow up the DFS data is likely to be identifying relevant trends earlier.

For pre menopausal ER- patients, for both DFS & OS, both the combined arm and the later conventionally timed chemotherapy arms seem superior to the early perioperative therapy alone arm and of these two the combined arm may be superior.

Table 3: **Four Year DFS & OS Percentages According to Treatment Group and Oestrogen Receptor Status. All patients Node Positive. Median follow-up time 42 months**

Treatment Group (percent ±SE)

		Early Perioperative Only	Combined	Later Conventionally Timed Only	P Value *
Premenopausal					
DFS	ER+ **	45±6	65±5	69±5	0.0003
	ER- ***	28±6	62±5	48±7	0.0005
OS	ER+	79±5	79±5	88±3	0.35
	ER-	51±7	66±6	60±7	0.31
Postmenopausal					
DFS	ER+	33±6	63±7	64±5	0.0001
	ER-	39±8	35±9	51±8	0.27
OS	ER+	79±6	84±5	86±4	0.51
	ER-	55±9	58±8	70±8	0.33

* Calculated with use of a two sided log-rank test for heterogeneity among treatment groups.

** ER+ = ≥ 10 (fmol)
*** ER- = 0-9 (fmol)

For initial interpretation of the data in attempting to identify helpful questions, it is reasonable to consider a non significant difference for OS data as important and probably representative if similar differences are seen in the DFS data and the latter are already significant. For example: the post menopausal ER+, DFS data, (p=0.0001), and the similar but non significant OS data for the same group (p=0.51). Beyond this interpretation must be cautious. The following questions arise.

i. From the evaluation of PC alone against a control no chemotherapy group in the N- patients, is PC effective and particularly so against ER- cells? If so, this might be a result of : a) these cells having a higher growth fraction immediately following surgery, and/or b) the possibility that any deleterious effect of surgery on residual tumour growth is either less important for N- patients (with a lower log burden) or it is counteracted by the PC. It should be noted that the estimated failure rate of these patients is still 23% at four years. Clearly, even for this group better therapy programmes are essential and urgently needed.

ii. Does the PC compromise the efficacy of subsequent (delayed) conventionally timed six cycles of CMFp in premenopausal N+ (large residual disease) patients? The data is persuasive for both DFS & OS. If the PC acts best on an ER- cell population (possibly because of a higher growth fraction), as seems to be the case for both N- and N+ premenopausal patients), a plausible explanation might be simply early killing or removal of sensitive cells. Thus, the combined therapy partly reduces the sensitive cell fraction such that by the time the delayed CMFp is commenced, the residual tumour burden has a higher fraction of resistant cells. Plausibly, this change in kinetics could alter the delayed CMFp efficacy. Alternative explanations include a reduction in total drug dose that occurs for the delayed CMFp when given after PC as distinct from when given alone (82.0 at least 80% of full CMF dose compared with at least 80% of full CMF dose, p=0.02) (Ludwig Breast Cancer Study Group 1988 (a)); and an immunosuppressive effect of the PC. The fact that PC is more effective in ER- N- than ER+ N- patients suggests that the first "kinetic" mechanism might be operating.

iii. Does the addition of PC fail to improve (or even worsen) the effect of a delayed conventionally timed six CMFp cycles regimen? This appears to be the case.

iv. In pre menopausal patients, is the addition of PC to CT of no value in ER+ patients, and deleterious in ER- patients? If true, similar explanations as those suggested for large residual tumour burdens probably apply.

This suggests that perhaps PC should be avoided in N+ ER- and/or large tumour burden patients. At present, the latter are identified by their nodal status (N+10) which is usually only discovered after the time during which PC is given.

v. In post menopausal ER+ patients, does the PC in large tumour burden patients compromise the efficacy of subsequent CT? If so, then this kinetic effect seems more important than any Tamoxifen-CMFp interaction. Either the PC compromises the efficacy of the CT CMFp and/or it augments a deleterious CMF-Tamoxifen interaction. Indeed, concurrent use of Tamoxifen and CMFp may well be the worst way of using both of these therapies for the same patient. Tamoxifen is known to hold up cells in G-1 phase of the cell cycle making it more difficult for CMFp to generate cell kill. As this hold up is likely to occur for the ER+ cell fraction and as PC could have reduced the size of residual sensitive more rapidly cycling ER- cells, there could be a dual compromise of CT therapy if Tamoxifen is added and PC is given first as in this group in Trial V.

vi. To what extent is the post menopausal data which demonstrates a benefit, a consequence of Tamoxifen effects on ER+ tumours? The pre menopausal patients seem to have benefited when CT is given alone rather than as part of the combined therapy. If this were so, then the postmenopausal patients with near identical DFS & OS for the patient group with ER+ tumours and the two Tamoxifen containing arms may be receiving the greater part of their benefit from the Tamoxifen. This is consistent with the overview data which demonstrated the effectiveness of Tamoxifen in post menopausal patients but little clear benefit of CMF in post menopausal patients as a group (Early Breast Cancer Trialists' Collaborative Group, Henderson et al, 1988).

vii. Does prior PC in post menopausal ER- patients compromise the efficacy of subsequent CT + Tamoxifen? The evidence for this is not strong, but the implications if true are important. If true this is in marked contrast to pre menopausal ER- patients where the combined regimen is apparently the best. This implies that the concurrent use of Tamoxifen & CMFp in the post menopausal ER- patients is possibly compromised by prior PC and apart from the kinetic arguments advanced above is difficult to explain.

When Trial V was commenced it was hoped that PC would be shown to be effective in N- patients. This is the case and the therapy has advantages - particularly in terms of patient tolerance. It would be more valuable if ER status were known before any major surgical intervention, as ER- patients (N+ or N-) seem to particularly benefit from early CMF. If they are found to be N+ and to have a low tumour burden, longer, ie additional CT with CMFp, is probably advantageous.

It was also hoped that Trial V would throw some light on the theory suggesting that spontaneous mutations may generate drug resistance. In the group where quite probably the only clear test of this is made (viz premenopausal, N+, ER-), the data strongly suggest that the combined arm is superior. Although such a result could be a consequence of therapy duration, the best corresponding group in this trial testing therapy duration is premenopausal N+, ER + where there is no difference between six and seven cycles of CMF. Thus the data are consistent with the PC being superior per se because of its timing rather than because it adds an extra cycle to the duration of therapy.

A New Mechanism for Understanding Metastases.

It has been long assumed that metastases i.e. tumour with the phenotype of a preexisting known "primary" tumour, result from cell transfer. Current knowledge provides a basis for alternative mechanisms and it is time to consider the implications of these for therapy. It seems plausible that in at least some circumstances, apparent metastases could result from altered phenotype of cells in the tissue where metastases are seen. This concept of recruitment or transformation of cells is fundamentally different from the current dogma which only considers cell transfer as the basis for metastasis. Thus, tumor seen in axillary nodes in a patient with breast cancer could be a consequence of phenotype change of cells in the node rather than a result of a mechanical arrest. The patient who has local recurrence in the breast following lump excision, could be exhibiting the results of a molecular process occurring systematically. It is simply more easily detected in the breast.

This concept more readily allows understanding of cancer as a systemic process and it also allows incorporation of current molecular biology, oncogene, and growth factor knowledge. It allows an understanding of how cancer cells can develop even after a primary tumour is removed. Current therapies have never done anything to correct the mechanism allowing the tumour to occur as they have largely concentrated on cell kill rather than cell control. This must change for further progress, as even if the cell free or recruitment mechanism operates in only 1% of metastases, its implications for therapy and for understanding the biology of tumors are broad.

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GROWTH CONTROL OF NORMAL AND MALIGNANT BREAST EPITHELIUM

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INTRODUCTION

In this presentation we review the multiple roles of both steroidal (primarily estrogen) and polypeptide regulators of mammary epithelial cell growth. Effects of both classes of hormones are complex and involve multiple interactions with non-malignant components. Estrogens induce growth regulatory polypeptide growth factors which regulate hormone dependent breast cancer. Progression of hormone dependent breast cancer to hormone independence may involve multiple genetic mechanisms of oncogene activation, loss of the estrogen receptor, or loss of hormone responsivity of other gene products. Initial carcinogenesis and progression of mammary epithelium to cancer probably also requires both proliferative stimuli (estrogen, polypeptide growth factors) and genetic damage, leading to qualitatively different hormonal responses (homorne responsive cancer). One goal of future therapies is to interfere with necessary activated or induced components of malignant progression such as oncogenes or polypeptide growth factors receptor systems. Alternatively, temporary perturbation of growth rate or growth fraction may be advantageously combined with traditional cytotoxic chemotherapy leading to increased efficacy.

ENDOCRINE, AUTOCRINE AND PARACRINE MECHANISMS OF GROWTH REGULATION

The clinical picture of malignancy depends on interactions of inherited genetic factors, exposure to chemical carcinogens, damaging radiation, oncogenic viruses, and mitogenic hormones, and other promotional agents (Bishop 1987). Experimental animal model systems (Welsch 1987) have allowed considerable insight into the mechanisms at work in the action of each of these components; however, the exact etiology of any human cancer is not fully established. The seminal work by Huggins (Huggin 1940) linking testicular secretions (androgen) to prostatic carcinoma and by Beatson (Beatson 1896) linking ovarian secretions (estrogens) to breast carcinoma represented critical insights into endocrine dependent neoplasia. Here, we will review the mechanisms of action of systemic estrogen in the human breast cancer process. We will also explore mechanisms of loss of endocrine control of experimental and clinical breast cancer, commonly observed following systemic therapy (Lippman 1983).

Loss or absence of estrogenic control of breast cancer growth during malignant progression implies the existence of other growth control processes which take over in its place as estrogens are essential for normal mammary epithelial growth. Recent work on locally acting, diffusable growth regulatory substances known as growth factors has provided a model for how additional growth controls might be exerted on mammary epithelial cells ranging from normal, to hormone dependent intraductal malignancies, to locally invasive malignancies, to metastatic tumor deposits, and finally to states of resistance to hormonal and chemotherapeutic agents (Dickson & Lippman 1986). In some cases, these growth factors are autostimulatory or "autocrine". In addition, a number of dominantly acting, cancer-associated genes, known as oncogenes (Pardee 1987; Slamon et al 1984), have recently been described. Some of these oncogenes encode growth factors or their receptors. Others appear to code for defective, cell membrane bound growth factor receptors which are enzymatically active even though they lack extracellularly exposed ligand binding sites. One oncogene codes for a protein with homology to nuclear receptors for steroid and thyroid hormones. Still other oncogenes appear to act distally on growth regulatory pathways including modulation of transcriptional complexes in the cell nucleus (Studzinski et al 1986; Bohmann et al 1987).

Genetic events which evoke the malignant phenotype probably involve activation of dominant oncogenes directly contributing to the process of transformation and inactivation of dominantly acting cancer suppressive genes. The mutation of cellular protooncogenes (all of which are putatively functional in physiologic growth control of normal tissue) to yield highly active oncogenes is extremely important in chemical-and radiation-induced carcinogenesis (see Bishop 1987 for review). Malignant progression of breast cancer through its various stages probably involves multiple elements including underlying genetic predisposition, mutation, and mitogenesis in response to estrogens, growth factors, and overexpressed growth factor receptors (Dickson et al 1987; Cline et al 1987). On a cellular level, the actual mechanisms involved in malignant progression remain conjectural. While the entire tumor could undergo progressive, malignant changes, this is not the most likely scenario. Rather, the bulk of observations suggest that subpopulations of genetically unstable cancer cells continuously arise within the tumor. Further genetic events in these subpopulations may create cells which tend to survive and overtake other less progressed tumor and normal cells. Surviving subpopulations are modulated by selective pressures: host defenses, competition for nutrients, survival after treatment with chemo-hormonal therapeutic agents and altered environment after metastatic spread (Nowell 1986; Nicholson 1987).

Growth control processes in breast cancer are not limited to the malignant cells themselves. Cancer depends upon an intimate interrelationship with non-tumor tissues of the host. The cancer must thwart host immune surveillance and nourish itself as its mass increases (Prehn & Prehn 1987). The processes of angiogenesis (blood vessel invasion) and desmoplasia (stromal proliferation), commonly observed surrounding breast cancer, are probably involved to some extent in these processes (Folkman 1986). Soluble mediators of such processes are known as "paracrine" acting hormones. Some of these hormones (the FGF's, PDGF) may also be encoded for by oncogenes (Sporn et al 1985; Sporn & Roberts 1985). Development of metastatic potential reflected by passage across the basement membrane, local invasion, infiltration of blood vessels and lymphatics and reseeding in distant metastatic sites probably also involves both oncogene- and mitogen-mediated processes.

While estrogen is the most important endocrine influence for the development and mitogenic control of breast cancer, a major thesis of this review is that it acts by triggering production of locally acting hormones. What kinds of hormones might act in local autocrine or paracrine fashions? A well established system for the identification of mediators of growth control has been provided by rodent fibroblasts *in vitro*. Smith, Scher and Todaro, among others, identified "restriction points" in the cell cycle of "normal" (but immortalized) fibroblasts. Various polypeptide growth factors abrogate these restriction points, allowing the cell cycle to progress (Smith et al 1971). Platelet derived growth factor (PDGF) allows cells to pass a restriction point in early G_1. As PDGF acts to initiate the cell cycle, several genes known as protooncogenes are sequentially induced. Among these are *c-fos* and *c-myc*,in the nucleus and *c-ras* in the plasma membrane (reviewed in Heldin & Westermark 1984). Epidermal growth factor (EGF) or the related transforming growth factor (TGFα), acts later, while insulin like growth factor - I (IGF-I) also known as somatomedin C and other hormones still later in G_1. One growth factor may induce others, which act further along in the cell cycle. For example, human diploid fibroblasts treated with PDGF, EGF, or growth hormone secrete their own IGF-I. Secreted IGF-I is capable of self-stimulation to promote mitogenesis; anti IGF-I antibodies block growth hormone stimulation of DNA synthesis.

Cells transformed with various tumor viruses, oncogenes, chemicals, or radiation lose some requirements for exogenous growth factors and produce more of their own as reflected in decreased serum requirement (Cherington et al 1979; Bradshaw & Dubey 1984; Heldin & Westermark 1984; Zahn & Goldfarb 1986). Thus, malignant transformation was proposed to result from ectopic production of growth factors (Delarco & Todaro 1978; Sporn & Todaro 1980).

The ability of some cells to grow in colonies under anchorage independent conditions (growth suspended in agar or agarose) was correlated with their tumorigenicity or state of malignant "transformation" (Freedman & Shiu 1974). Four growth factor activities have been identified which together can reversibly induce this transformed phenotype of murine fibroblasts: PDGF, EGF (or TGFα), IGF-I (or IGF-II, a different somatomedin activity) and an additional growth factor, transforming growth factor β (TGFβ) (Sporn & Todaro 1980; Assoian et al 1984; Massague et al 1985). An important aspect of TGFβ's action as a transforming agent appears to be its induction of basement membrane components, such as collagen and fibronectin (Ignotz & Massague 1986) and the *c-sis* protooncogene (Leof et al 1986). While results using anchorage independent growth assays suggest that these growth factors are involved in cancer growth control, little direct evidence for an *in vivo* role in tumor growth has yet emerged.

The details of cell cycle regulation for epithelial cell cycles are unknown. It is clear that some normal human mammary epithelial cells require a glucocorticoid, insulin, EGF, PGE_1 (a prostaglandin), transferrin, (the iron-carrying serum protein), and an incompletely defined pituitary component(s) to proliferate in serum free medium (Hammond et al 1984). In contrast to the fibroblast model, TGFβ is a growth inhibitor for many types of normal and malignant epithelial cells, including breast, (Tucker et al 1984; Roberts et al 1985). While some of the same growth factors may facilitate traverse of the cell cycle in both fibroblasts and epithelial cells, control of anchorage independent growth may involve another growth factor(s). A candidate for such an epithelial transforming growth factor is provided by the work of Halper and Moses (Halper & Moses 1983). They have identified an adrenal carcinoma cell line (SW13) which is extremely sensitive for anchorage independent cloning to a mitogen found in epithelial cancers or cell lines. Basic pituitary FGF also stimulates cloning of SW13 and their epithelial cancer derived growth factor may be a new member of the FGF family. Using an independent model system, SV40T oncogene transfected, immortalized human mammary epithelial cells, TGFα, EGF, and FGF can also be shown to have transforming activity (Clarke et al 1988; Valverius et al 1988). Finally, in MCF-7 human breast cancer cells, estrogens are capable of inducing anchorage independent growth (Lippman et al 1976; Sutherland et al 1986). Estrogenic induction of growth factors and elaboration of basement membrane components contributes to control of the malignant phenotype.

Regulation of neoplastic growth by estrogens is presumably a modified remnant of normal regulatory mechanisms for mammary epithelial proliferation and differentiation. While estrogens are mitogens for both normal and malignant breast epithelium, the hypothalmus-pituitary axis is indirectly in control of ovarian estrogen secretion by virtue of GnRH and gonadotropin stimulation (Ross et al 1981). In addition, the pituitary gland (or other organs) may also secrete other direct or indirect acting mitogens (Eidne et al 1985; Wilding et al 1987) such as IGF-II, FGF, or LHRH. Studies of murine model systems show that estrogen can control breast tumor growth by inducing pituitary synthesis and secretion of prolactin. Sirbasku has employed the term "estromedin" for other analogous, but still hypothetical, estrogen induced, endocrine acting mitogens (Ikeda et al 1983). Estrogen might also act by allowing breast cancers to overcome growth inhibitory agents in their environment or by synergy with other stimulatory agents (Lykkesfeldt & Brand 1986; Devleeschouwer et al 1987; Soto & Sonnenschein 1987). These interacting components could be serum derived, produced by the cancer itself, or produced by nearby tissues. In fact, there has been a surprising lack of evidence for direct estrogenic effects on normal mammary epithelium. Studies of hormonal control of breast cancer have been facilitated by the availability of cancer cell lines, usually derived from pleural or ascites fluids of patients. Several estrogen responsive lines exist including MCF-7, T47D, MDA-MB-134, ZR-75-1, PMC42 and CAMA-1 (Brooks et al 1973; Lippman et al 1976; Engle & Young 1978; Weischselbaum et al 1978; Edwards et al 1980; Soule & McGrath 1980; Butler et al 1981; Chablos et al 1982; Leung et al 1982; Darbre et al 1983; Katzenellenbogen et al 1983; Natoli et al 1983; Page et al 1983; Simon et al 1984; Berthois et al 1986; Reiner & Katzenellenbogen 1986; Osborne et al 1987), the best characterized of these being MCF-7 (Brooks et al 1973). MCF-7 has an absolute requirement for estrogen for tumor formation in the athymic (nude) mouse (Soule & McGrath 1980). Experimental findings obtained using cell lines must be regarded with caution.

After years in laboratory culture, subclonings, and assorted selective pressures, one can only hope that data derived from these cell lines will prove relevant to understanding of tumorigenesis in vivo. This hope can only be fulfilled by eventual in vivo clinical verification.

Initial studies on in vitro hormone responsivity of MCF-7 cells produced disparate reports concerning the growth responses to estrogen. We and others succeeded in demonstrating receptors for estrogens (Brooks et al 1973; Lippman et al 1976) and direct proliferative responses to physiologic doses of 17β-estradiol (E_2) in vitro (Lippman et al 1976; Weischselbaum et al 1978; Chablos et al 1982; Darbre et al 1982; Darbre et al 1983; Katzenellenbogen et al 1983; Natoli et al 1983; Page et al 1983; Simon et al 1984; Berthois et al 1986) and in vivo in the nude mouse (Engle & Young 1978; Soule & McGrath 1980). A number of other groups failed initially to observe these responses (Edwards et al 1980; Butler et al 1981; Devleeschouwer et al 1981; Lykkesfeldt & Briand 1986; Soto & Sonnenschein 1987). One problem appears to have been that some groups were working with an incorrectly identified or contaminated MCF-7 cell line (Osborne et al 1987). Other discrepancies have now been largely resolved with a more complete understanding of relevant variables in culture conditions. Serum is a rich source of estrogenic compounds, including sulfate conjugates, which must be removed to observe maximal effects of exogenous estrogen in vitro (Butler et al 1981; Darbre et al 1983; Page et al 1983). Furthermore, a component in phenol red, commonly present in culture medium as a pH indicator, can produce estrogenic effects (Berthois et al 1986; Nelson et al 1987). Finally, growth factors in the cellular environment (particularly representatives of the insulin family) can critically modulate estrogen responses (Butler et al 1981).

A well differentiated estrogen responsive breast cancer cell line, PMC42, has recently been described. Monoclonal antibodies prepared against surface antigens of this cell line cross react with intraductal (non-invasive) breast cancer specimens (Whitehead et al 1984; Dempsey et al 1986). Numerous estrogen independent breast cancer lines also exist (Engle & Young 1978). While existing cell lines can be sorted according to their estrogen receptor status, nearly all were derived from metastatic sites in patients and are fully malignant in that sense. Thus, controls on metastatic behavior have been difficult to address in cell culture systems.

We next turn to data showing that estrogens directly interact with receptor-containing breast cancer cells to modulate gene expression and phenotypic properties. We suggest that polypeptide growth factors are common mediators of growth control for normal breast, estrogen regulated breast cancer and hormone independent breast cancer. While stressing direct effects of estrogens on cancer cells, we do not wish to suggest that growth control of tumors in vivo may not be a more complex phenomenon resulting from interactions among other cell types, hormones, proteases, and basement membrane components.

Estrogens induce increased activity of a large number of enzymes and other proteins involved in nucleic acid synthesis in isolated breast cancer cell lines including DNA polymerase, the c-myc protooncogene (Dubik et al 1987), thymidine and uridine kinases, thymidylate synthetase, carbamyl phosphate synthetase, aspartate transcarbamylase, dihydroorotase, glucose 6-phosphate dehydrogenase, and dihydrofolate reductase (Edwards et al 1980; Aitken & Lippman 1983; Aitken & Lippman 1985; Dickson et al 1987). Physiologic concentration of estrogen stimulate DNA synthesis by both scavenger and *de novo* biosynthetic pathways. Estrogen regulates thymidine kinase and dihydrofolate reductase at the mRNA level (Cowan et al 1982; Kasid et al 1986). Regulation of thymidine kinase mRNA has been shown to occur at the transcriptional level. Increases in global transcription appear to be tightly coupled to estrogen action (Aitken et al 1985). The existence of "second message" regulatory systems in the growth induction process is also possible but has not yet been proven. In MCF-7 cells, estradiol-induced stimulation of phosphotidyl inositol turnover to generate diacylglycerol and inositol-trisphospate occurs with an exceptionally long lag time (Freter et al 1988). In other polypeptide growth factor or protease-induced model systems, this metabolic effect is rapid (within minutes as opposed to hours for estrogenic effects) and tightly coupled to growth control (Nishizuka 1984; Carney et al 1985). Thus, phosphatidyl inositol turnover, with its associated stimulation of Ca^{++} fluxes by inositol-trisphosphate and of protein kinase C by Ca^{++} and diacylglycerol and could serve as a metabolic mediator of mitogenic effects of estrogen-induced growth factors and/or protease. One potential target for protein kinase C is the Na^{+}/H^{+} antiporter system. The Na^{+}/H^{+} antiporter is activated in a number of mitogen triggered proliferation systems. Inhibition with the antidiuretic amiloride prevents proliferative responses in some systems (Davis & Pouyssegur 1986). Protein kinase C is not an oncogene. However, its expression can lead to disordered morphology of fibroblasts (Housley et al 1988; Persons et al 1988).

Ornithine decarboxylase (ODC) is covalently linked to cellular membranes through inositol. This bond is broken by a phosphatidylinositol-specific phospholipase C, activating the ODC enzyme (Mustelin et al 1987). ODC activity is associated with induction of proliferation in numerous systems including breast cancer (Manni et al 1986). The actual contribution to growth control by any of these potential mediators (protein kinase C, ODC, Ca^{++}, or Na^{+}/H^{+} antiporter) remains to be determined.

Progesterone receptor is also induced by estrogen (Horwitz & McGuire 1978). Estrogen induces the progesterone receptor at the mRNA level (Nardulli et al 1988; Read et al 1988). Progestins are generally growth inhibitory for human breast cancer while inducing a specific protein of 48 kDa (Chablos & Rochefort 1984). The induction of the progesterone receptor by estrogens is generally coupled to functional growth regulation by estradiol *in vivo* and *in vitro*. Thus, progesterone receptor content in breast tumors is used as a marker for estrogen and antiestrogen responsiveness of tumors (90). Widespread exceptions do, however, exist *in vitro* to the coexpression of these two receptors (58,86) (for example T47D and MD-MB-134) and in some patient tumors (Lippman 1985).

Estrogens and antiestrogens alter the cellular synthesis and/or secretion of several other proteins whose role in growth control is unclear. These proteins include various plasminogen activators and collagenolytic enzymes. These proteases may contribute to tumor growth and progression by allowing the tumor to digest and traverse encapsulating basement membrane (Butler et al 1979; Huff & Lippman 1984; Liotta et al 1986; Terranova et al 1986). Proteases may also facilitate release of mitogenic growth factors like IGF-I (somatomedin C) from carrier proteins, process inactive precursor growth factors and proteases to active species (Kaufman et al 1977) or interact directly with their own cellular receptors (Neufeld & Ashwell 1980; Stopelli et al 1986). In addition breast cancer cells secrete proteins of 24 kDa (Ciocca et al 1983), 52 and 160 kDa (Westley & Rochefort 1980; Westley & May 1987), 37-39 kDa, 32 kDa (Bronzert et al 1987; Sheen & Katzenellenbogen 1987) and 7 kDa (initially identified by detection of an estrogen induced mRNA species termed pS2) (Jakolew et al 1984; Nunez et al 1987). Four other mRNA species termed pNR 1-4 (May & Westley 1986), and the cytoplasmic enzyme LDH (Burke et al 1978) are also under estrogen regulation. The 52 kDa glycoprotein, one of the major secreted proteins, has cathepsin D-like activity in purified form and is also mitogenic for MCF-7 cells in vitro (Vignon et al 1986; Capony et al 1987). The roles of the 160, 37-39, 32, 24, and 7 kDa proteins are unknown at present, but the 160, 52, and 7 kDa secreted proteins may be disassociated from in vitro estrogen and antiestrogen modulation of MCF-7 cells growth using two MCF-7 clonal variants (Bronzert et al 1984; Bronzert et al 1985; Davidson et al 1986). These three protein species are decreased by antiestrogen to the same extent in both MCF-7 and LY2, the latter being a stable antiestrogen resistant variant of MCF-7. In I-13, an MCF-7 clonal variant which is growth arrested by physiologic concentrations of estrogen, the same three proteins are induced to the same extent as in MCF-7. These observations suggest that a significant alteration in secretion of these major proteins has no impact on growth (at least in vitro) in LY2 or I-13.

Estrogens induce the cell surface "receptor" or binding protein for laminin in MCF-7 cells (Albini et al 1986; Thompson et al 1988). The laminin receptor mediates attachment of cells to basement membrane laminin (Liotta et al 1986; Terranova et al 1986) and contributes to invasiveness by tumor cells. Estrogen treatment of MCF-7 cells increases I^{125}-laminin binding, cell attachment to artificial, laminin-coated membranes, and the migration of the same cells across an artificial membrane toward a diffusible source of laminin (Thompson et al 1988). Estradiol treatment of MCF-7 cells also induces rearrangements of cytoskeletal and adhesion structures (Sapino et al 1986) and alterations in the plasma membrane microvilli as observed by scanning electron microscopy (Vignon et al 1983).

We next consider breast cancer related growth factors and the evidence supporting a pathophysiologic role for them in the growth and malignant progression of mammary cells.

TGFa (transforming growth factor α) was initially described as a product of virally-transformed rodent fibroblasts by Delarco, Todaro, Sporn and Roberts (Delarco & Todaro 1978; Roberts & Sporn 1986). TGFα has now been found in many proliferating normal and malignant human tissues. EGF was initially characterized from rodent salivary glands by Cohen (Cohen 1962), but is now known to be more widely expressed in human tissues. The human form was originally known as urogastrone, a placental product. TGFα is known to exist in 25 kDa, 21 kDa, and 17-19 kDa precursor forms (Bringman et al 1987) and commonly processed to a 7 kDa form. EGF appears to be processed from a very large precursor form (130 kDa) with multiple polypeptide products (Scott et al 1983). EGF, TGFα, and a related protein from vaccinia virus form a functional family of growth factors which apparently utilize the EGF receptor to carry out their many functions (Carpenter et al 1983).

Human breast cancer cells in culture produce TGFα. Cell lines secrete stimulatory factors for MCF-7 and murine 3T3 fibroblast monolayer cultures as well as "transforming growth activity" (TGF) determined by stimulation of anchorage independent colonies of rodent NRK and AKR-2B fibroblasts in soft agar culture (Nickell et al 1983; Salomon et al 1984; Bates et al 1986; Dickson et al 1986; Bates et al 1988). Some breast cancer cell lines produce a 30 kDa molecular weight species of transforming activity for NRK fibroblasts. This species comigrates chromatographically with a peak of MCF-7 autostimulatory activity and is the principle species of EGF receptor competing activity (Bates et al 1986; Dickson et al 1986). Antisera specific to TGFα react with this species (Perroteau et al 1986). Thus, this activity is related to TGFα but appears to be significantly larger than the cloned and sequenced 6 kDa species from transformed rodent fibroblasts (Derynck 1984). It is not yet certain if this protein is related to the 17-19 kDa TGFα precursor protein observed in transformed fibroblasts (Ignotz et al 1986; Bringman et al 1987; Gentry et al 1987), whether it is modified by glycosylation, palmitoylation (Bringman et al 1987), or if it is the product of alternative mRNA splicing. The precursor species is though to be membrane bound in cell lines which express it (Ignotz et al 1986; Bringman et al 1987; Gentry et al 1987). It cannot as yet be ruled out whether breast cancer derived TGFα may be the product of a novel TGFα-related gene. The 30 kDa TGFα-like species is induced by estrogen treatment of estrogen receptor positive MCF-7, T47D, and ZR-75-1 cells from 2-14 fold depending on cell type and culture conditions (Bates et al 1986; Dickson et al 1986; Dickson & Lippman 1986; Perroteau et al 1986; Dickson et al 1987; Salomon et al 1988). An expected 4.8 Kb TGFα mRNA species has been detected in MCF-7 and other human breast cancer cell lines and confirmed by RNAse protection methodologies. TGFα mRNA and protein can be detected in breast tumors (Derynck et al 1987; Bates et al 1988) ranging from low to high estrogen receptor content. No correlation of TGFα mRNA expression was observed with estrogen receptor status. At least 70% of biopsy samples of adenocarcinomas contained TGFα mRNA (Bates et al 1988). The estrogen receptor negative, Hs578T carcinocarcinoma cell line does not contain detectable levels of TGFα protein or its mRNA but many other estrogen receptor negative cell lines do (Bates et al 1986; Bates et al 1988).

TGFα mRNA is induced in 6 hrs in MCF-7 cells treated with estradiol (Bates et al 1988). Similar observations have been made in hormone dependent mouse mammary tumors (Liu et al 1987). Using antibodies directed against either TGFα or its receptor (the EGF receptor) we have noted growth suppression of MCF-7 and other human breast cancer cell lines grown as anchorage independent colonies or as estrogen stimulated, high density monolayer cultures (Bates et al 1988). Thus, TGFα is a likely autocrine growth factor in breast cancer. It has also been detected in the urine of patients and nude mice bearing breast and other human tumors (Kimball et al 1984; Stromberg et al 1987). Thus it may be provide a marker for tumor burden or disease progression. Detection of urinary TGFα has been complicated by the presence of very high levels of EGF-related growth factors present even in normal control urine (Kimball et al 1984).

The insulin family of growth factors is a complex group of cross reacting ligands further complicated by multiple receptors and serum-borne binding proteins.

Insulin is a two chain disulfide linked growth factor, processed from a single gene product whose primary site of synthesis is the pancreas. In contrast, the single chain (uncleaved, 7.5 kDa size) IGF-I and IGF-II growth factors (somatomedins) are synthesized by many body tissues (including liver) and are under entirely different hormonal regulation (Davidson et al 1987; Hynes et al 1987). Several other growth factors including relaxin and lentropin (which controls lens fiber formation) appear to be members of an even larger insulin-related family (Beebe et al 1987). Alternative splicing of mRNA of the insulin-like growth factors (particularly IGF-I (Rotwein 1986)) also contributes to the complexity of members of this diverse family.

IGF-I is mitogenic for some breast cancer cells in culture (Furlanetto & DiCarlo 1984; Huff et al 1986). An IGF-I related species is secreted by all human breast cancer cells examined to date (Baxter et al 1983; Huff et al 1986). After partial purification from MCF-7 cell conditioned medium this growth factor comigrates on gel exclusion chromatography with authentic human serum-derived IGF-I. Acid ethanol extraction is required to partially disrupt a high molecular weight form of the growth factor. A complex series of IGF-I-cross reacting mRNA species are also detected with northern blot analysis using a cDNA probe to authentic IGF-I (Huff et al 1986). However, none of these mRNA species are identifiable as authentic IGF-I upon nuclease protection analysis of mRNA (Yee et al 1988). Complex species of IGF-I cross hybridizing mRNA's have been previously described for the human fetus (Han et al 1987). In phenol red-free medium, there is a 3- 6-fold induction of IGF-I-like growth factor with estrogen, TGF*α*, EGF, or insulin treatment (Huff et al 1988a). Secretion of IGF-I related factors is inhibited by antiestrogens (in phenol red-containing medium) TGF*β*, and glucocorticoids. While growth hormone is a strong stimulus for IGF-I production by liver, fibroblasts, and other normal tissues it is without effect on production of IGF-I-like growth factors by MCF-7 breast cancer cells (D'Ercole et al 1980; Clemmons & Shaw 1983; Jansen et al 1983; Clemmons & Van Wyk 1986; Huff et al 1988a). IGF-I-related polypeptides are secreted by fibroblasts and smooth muscle and contribute to autocrine growth control in these cell types (Clemmons & Shaw 1983; Clemmons & Shaw 1986; Clemmons & Van Wyk 1986). It remains to be seen whether IGF-I produced by breast cancer acts primarily on breast cancer itself in an autostimulatory mode or on surrounding stroma to promote chemotaxis and growth. Alternatively, it may be true that breast cancers induce surrounding mesenchyme to produce IGF activities which may function to stimulate the mesenchyme or the breast epithelium. Recent studies have shown that an antibody which blocks the IGF-I receptor (Flier et al 1986) is capable of inhibiting MDA MB-231 breast cancer cloning in vitro (Rohlik et al 1988) and tumor growth in vivo (Arteaga et al 1988) suggesting either autocrine or paracrine stimulation of breast cancer growth by this or a related peptide.

At the present time insufficient information is available to fully evaluate the structure, regulation, and function of breast cancer-derived IGF-I-related proteins and compare them to those produced by embryonic and adult tissues (Nissley & Rechler 1984; Van Wyk 1984). Since insulin synergizes with estrogen in promoting growth breast cancer cell lines in vitro and in vivo in the nude mouse it is possible that somatomedins principally act by interacting with estrogen to promote breast tumor growth (Huff et al 1988b; Van der Burg 1988).

It has also been reported that an IGF-II-related gene product(s) are produced by normal and malignant tissue (Cullen et al 1988). IGF-II appears to bind to multiple receptors (insulin, IGF-II, and IGF-I). All of these receptors, including the IGF-II receptor have been detected in human breast cancer (Furlanetto & DiCarlo 1984; DeLeon et al 1988) as well as lactating states of normal breast (Dehoff et al 1988). While IGF-II interaction with IGF-I receptors may stimulate cellular response, IGF-II receptor may be primarily involved in IGF-II degradation. The IGF-II receptor is a multifunctional protein, previously described as the mannose-6-phosphate receptor for lysosomal enzymes (Harri et al 1987; Morgan et al 1987). IGF-I mRNA-like species have been recently reported in other human tumors: lung, colon, and liposarcoma (Minuto et al 1986; Tricoli et al; Kiesse et al 1987), IGF-II has been observed to be overproduced in Wilms tumor (Reeve et al 1985). Somatomedins appear to be among the most ubiquitous growth factors, produced by nearly all normal tissues (Nissley & Rechler 1984; Van Wyk 1984; Rechler & Nissley 1986) and found in the blood (Stiles et al 1979) and urine (Hizuka et al 1987).

Transforming growth factor beta$_1$ (TGFβ_1) is a 25 kDa polypeptide initially purified from platelets and various normal tissues. It is required (along with other growth factors) for full induction of the transformed phenotype in fibroblasts. It is also produced autonomously in fibroblasts transformed by oncogenes (Sporn et al 1987). TGFβ_1 is a member of a multi-gene family which includes at least three other TGFβ's Mullerian inhibiting substance, inhibins and activins (Carter et al 1987), a T cell suppressor factor (Wrann et al 1987) and a Drosophila morphogenesis-controlling gene known as decapentaplegic (Padgett et al 1987). In contrast to TGFα and many other growth factors, TGFβ_1 is growth inhibitory and/or differentiating-promoting for most epithelial cells (Tucker et al 1984; Roberts et al 1985; Chakrabarty et al 1988). For example, it inhibits myogenic differentiation (Massague et al 1986; Olson et al 1986), it also inhibits normal hepatocyte growth more extensively than malignant liver cell growth, and it prevents dedifferentiation of other epithelial cell types (McMahon et al 1986; Rizzino 1987). In addition, it stimulates differentiated behavior of vascular smooth muscle (184) and normal breast epithelial cultures (Walker-Jones et al 1988).

In normal mammary epithelial cells TGFβ induces synthesis of milk fat globule antigen. In addition, growth of these cells in culture is arrested and the morphology is markedly altered; TGFβ changes the cobblestone epithelial appearance to an elongated spindle shape (Walker-Jones et al 1988). TGFβ_1 also appears to be extremely potent in vivo in the neonatal mouse. Implants of TGFβ_1 in slow release capsules near developing mammary ducts result in complete cessation of mammary ductal development. No effects of TGFβ_1 are seen on surrounding stromal tissue or on more distant mammary glands (Silberstein & Daniel 1988). TGFβ_1 may play a role, along with other hormones such as estrogen and growth factors (such as EGF or TGFα) (Vonderhaar 1988) in the delicately balanced process of mammary development. TGFβ_1 (along with a plethora of other growth factors) is also a component of human milk (Noda 1984).

Breast cancer, like normal breast epithelium, is inhibited by TGFβ_1. TGFβ_1 may be an autocrine inhibitory type substance (chalone) (Wang & Hsu 1986) in breast cancer. Breast cancer cells have been shown to contain and secrete a TGFβ-related activity (Derynck et al 1985; Roberts et al 1985; Bates et al 1986; Knabbe et al 1987; Knabbe et al 1988). The activity binds to TGFβ receptors, transforms AKR-2B and NRK fibroblasts, is immunoprecipitated by TGF-beta antiserum, and comigrates on gel exclusion columns with platelet-derived TGFβ_1 (Knabbe et al 1987). All breast cancer cells lines reported express the expected 2.5 Kb mRNA species (Wang & Hsu 1986; Derynck et al 1987; Knabbe et al 1987). TGFβ_1 secretion is inhibited by treatment of MCF-7 cells with estrogen and insulin (Knabbe et al 1987), but growth inhibitory antiestrogens and glucocorticoids strongly stimulate its secretion. Intracellular TGFβ does not appear to change in concentration following treatment with mitogens or growth inhibitors (Knabbe et al 1987). TGFβ_1 from antiestrogen induced MCF-7 cells strongly inhibits the growth of an estrogen receptor negative cell line MDA-MB-231. This growth inhibition was reversed in the presence of a polyclonal antibody directed against native TGFβ (Knabbe et al 1987). Since breast cancers exist as mixtures of estrogen receptor positive and negative tumor cells (Greene et al 1984; King et al 1985) and breast cancers may not become TGFβ_1 unresponsive as they become antiestrogen unresponsive, TGFβ_1 may act in tumors with such mixed cell populations to make antiestrogen more effective than might otherwise be expected based only upon blockade of estrogen action (Knabbe et al 1987). In LY2, an MCF-7 variant stepwise selected in vitro for antiestrogen resistance, TGFβ_1 is no longer induced by antiestrogen, but the cells still retain the TGFβ receptor and response. Neither the mechanism of TGFβ induction in MCF-7 cells nor its loss in LY2 cells is fully defined, but it is not at the regulation of steady state mRNA for TGFβ_1. Conversion of a latent form to an active form of TGFβ may contribute (Knabbe et al 1987). In contrast to other cell types (Lawrence et al 1984; Hizuka et al 1987), there is significant active TGFβ present in breast cancer conditioned medium. The biochemical details of the conversion of a secreted inactive to active TGFβ remain to be elucidated.

PDGF is a heterodimeric protein of approximately 30 kDa, which is found in high concentrations in platelets. PDGF-like related growth factors are produced by a variety of transformed murine fibroblast lines and by some human tumors of diverse origins. The v-sis oncogene is related to a PDGF B chain homodimer and can transform PDGF-receptor-expressing cell types (Heldin & Westermark 1984). Consequently, PDGF could subserve an autocrine role in such tumors. In tumors derived from cell types lacking the PDGF receptor, v-sis is not transforming and PDGF presumably functions in a paracrine mode to stimulate angiogenesis, stromal proliferation (desmoplasia) and chemotaxis and degranulation of monocytes and neutrophils (Ross et al 1986).

Transformation of fibroblastic cells with simian sarcoma virus (SSV) provides a model system for the function of PDGF in responsive cell types. The PDGF-B chain related protein encoded by the virus forms a homodimer and is secreted by the cell. Antibodies directed against PDGF have been reported to exert antiproliferative and antitransforming activity (Ross et al 1986). However, in many instances the PDGF is largely cell associated and presumably already bound to its receptor. Thus, anti-PDGF antisera have been only partially effective as antiproliferative reagents (Huang et al 1984). The subcellular origin or fate of PDGF remains to be fully characterized, however, immunoreactive PDGF has been observed in the cell nucleus (Yen et al 1987) in SSV-transformed cells. The PDGF encodes a short peptide sequence which is capable of directing the molecule across the nuclear pore complex (Lee et al 1987). It has been proposed that PDGF may exert autocrine growth control through both intracellular and extracellular mechanisms of action (Keating & Lewis 1988).

Many breast cancer cell lines secrete a PDGF-related activity detected by anchorage dependent growth stimulation of mouse 3T3 fibroblasts in the presence of platelet poor plasma. This is known as a "competency" assay for early mitogenic signals (Rozengurt 1986). 28 kDa and 16 kDa species were observed by immunoprecipitation of metabolically labeled MCF-7, MDA-MB-231 and other breast cancer cell extracts and medium. The 28 kDa species (the unreduced form) was biologically active after elution from non-reducing SDS-polyacrylamide gels, and its activity was blocked with anti-PDGF antiserum. Upon examination of poly A-selected mRNA from either cell line, transcripts of both PDGF A and B chains are observed (Rozengurt et al 1985; Betsholtz et al 1986; Bronzert et al 1987; Peres et al 1987). A and B chains are widely expressed in breast cancer and other cell lines (Roberts et al 1986; Folkman & Klagsburn 1987; Takehara et al 1987; Wahl et al 1987). While the B chain is homologous to the v-sis oncogene, the A chain is not known to have a retroviral oncogene homologue. The A and B chain share substantial sequence homology to each other (Yarden et al 1986) and the A chain shows evidence of alternative mRNA splicing (Tong et al 1987). It is not yet known how A and B chains assemble in breast cancer cells.

The fibroblast growth factors, like PDGF were initially classified as "competency" factors acting early in the G_1 phase of the cell cycle to stimulate the growth of mesenchymal cells. The members of the family include acidic and basic FGF (Esch et al 1985) (aFGF and bFGF), kaposi FGF (kFGF, also known as hst (for human stomach tumor oncogene) (DelliBovi et al 1987), and int 2 (Dickson & Peter 1987) (a mouse mammary cancer oncogene mentioned earlier). FGF_5 has recently been described (Zhan et al 1988). Other members of this family are likely to be described. A more distant homology also exists with interleukin 1 (IL-1)(Thomas et al 1985). bFGF is capable of acting as an oncogene when expressed in fibroblasts (Rogel et al 1988; Sasada et al 1988). It is not yet known how many classes of receptors exist for this diverse class of ligands. Both aFGF and bFGF bind a 140-210 kDa receptor and stimulate tyrosine phosphorylation of a 90 kDa protein (Coughlin et al 1988). A putative FGF receptor has recently been purified to homogeneity (Lee et al 1989).

FGF is required for normal mouse mammary cells to proliferate in culture. It is present in pituitary extract used for culture of mouse and human mammary myoepithelial and epithelial cells (Hammond et al 1984; Smith et al 1984). FGF is also a potent angiogenic substance (Thomas et al 1985). The FGF family of peptides is characterized by a binding site for heparin (Esch et al 1985). This has facilitated purification and may allow for strategies to interrupt or otherwise modulate FGF action through binding of various polyanionic substances such as suramin to this site (Coffey et al 1987). bFGF does not possess a signal peptide in its primary sequence (Abraham et al 1986), giving rise to hypotheses for unusual secretory pathways including intracellular binding to heparin proteoglycan and secretion as a part of the basement membrane (Esch et al 1985). Hst and FGF_5 are secreted (Zhan et al 1988).

Uncertainty exists as to the principle target of FGF in the normal mammary gland. Rudland has proposed that effects are restricted to myoepithelium and stroma (Rudland 1987) but Sirbasku has found that MCF-7 and T47D human breast carcinoma cells respond. A 60 kDa, heparin-binding FGF-related molecule is produced by human breast cancer cells in culture. Its function could include autocrine and paracrine effects (Halper & Moses 1987; Karey & Sirbasku 1988).

Growth factors and/or estrogen probably act in vivo in concert with other systemic mitogens to promote tumor growth. Shiu and coworkers have isolated a pituitary derived activity which potentiates the mitogenic effects of estradiol on MCF-7 cells (Swain et al 1986). One pituitary factor has already been identified as IGF-II (Dembinski et al 1985). In addition, pituitary derived GnRH may also directly interact with breast cancer to inhibit its proliferation (Shiu et al 1988) while prolactin is stimulatory for some cell lines (Eidne et al 1985).

MCF-7 cells in monolayer culture show growth regulation by a variety of lipid soluble hormones in addition to estrogen. These include glucocorticoids, iodothyronines, androgens and retinoids (reviewed in Biswas & Vonderhaar 1987). MCF-7 cells have receptors but are not growth stimulated by progesterone or vitamin D (Chablos et al 1980; Rochefort et al 1980; Lippman 1984). Progesterone induces a specific protein (Rochefort et al 1980) and can be growth inhibitory in vitro (reviewed in Eisman et al 1980). Other inhibitory hormones include somatostatin (Setyono-Han et al 1987) interleukins 1 and 6, tumor necrosis factor (TNF), and interferon (reviewed in Sporn & Roberts 1988). Receptors and metabolic effects, but little cellular growth response has been demonstrated for other hormones, such as growth hormone, glucagon and calcitonin (Lippman 1984). Finally, transferrin, a serum iron delivery molecule is required for proliferation of normal and malignant mammary cells (Hanover & Dickson 1985); its receptor is increased in estrogen independent breast cancer compared to estrogen dependent breast cancer (Tonik et al 1986). The multiplicity of growth modulatory hormones for in vitro breast cancer systems suggests the possibility that many serum borne or locally produced modulators of growth may play important regulatory roles in vivo. Alternatively, or additionally, growth factors with a similar spectrum of activities could be elaborated by the breast cancer cells themselves.

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STUDIES OF EGF-MEDIATED GROWTH CONTROL AND SIGNAL TRANSDUCTION USING THE MDA-MB-468 HUMAN BREAST CANCER CELL LINE

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EPIDERMAL GROWTH FACTOR RECEPTOR IN BREAST CANCER

Epidermal growth factor (EGF) is a mitogenic polypeptide affecting proliferation of a variety of cells. The tissue-specific actions of EGF are mediated through binding to a trans-membrane receptor glycoprotein (EGFR). The receptor is composed of an extracellular EGF binding domain, a transmembrane segment and an intracellular domain which has tyrosine kinase activity (Carpenter, 1987). Evidence for a role for EGF in growth control of mammary epithelial cells has come from a number of sources (Table 1) including the effect of EGF on development of mammary gland during pregnancy (Okamoto and Oka, 1984) and carcinogenesis of the breast in rodent systems (Kurachi et al, 1985). EGF is also a mitogen for many breast cancer cell lines in tissue culture (e.g. Fitzpatrick et al, 1986) and many breast tumors express high levels of the receptor protein as evidenced by EGF binding or immunohistochemical staining (Sainsbury et al, 1985). It is of interest that there is an inverse relationship between such EGFR expression and estrogen receptor (ER) status; it has been suggested therefore that, in ER-ve tumors, the EGFR may be involved in growth control.

The mechanism underlying the over-expression of the EGFR in breast tumors has not been elucidated in detail. However, unlike other tumor types which over-express the EGFR such as squamous carcinoma and glioblastoma (Ozanne et al, 1986; Libermann et al, 1985), the predominant mechanism is not based on receptor gene amplification. Although approximately 20% of breast tumors express elevated levels of EGFR, only approximately 2% have documented EGFR gene amplification. Since the cell of origin of ER-ve breast tumors is not established, and the level of EGFR expression in differentiating normal mammary epithelium is not known, it remains a possibility that the variability in expression may not be related to a mutational event in the carcinogenesis process, but rather to the expansion of a rare cell population which normally expresses high levels of EGFR.

TABLE 1. EGF: Role In Mammary Epithelial Development and Carcinogenesis

- necessary for rodent gland development during pregnancy (Okamoto and Oka, 1984)
- stimulates proliferation and morphological development of rodent tissue in *in vitro* systems (e.g. Vonderhaar and Kaklasi, 1986)
- involved in rodent spontaneous mammary tumor development (Kurachi et al, 1985)
- mitogen for a number of breast cancer cell lines in culture (e.g. Fitzpatrick et al, 1986)
- level of EGFR in biopsies related to prognosis in breast cancer; over-expressed in ER-ve tumors (Harris and Nicholson, 1988)
- overexpression in biopsies rarely associated with gene amplification

As an attempt to derive laboratory tissue culture models of this type of breast tumor, we screened a series of breast cancer cell lines for EGF binding capacity. We identified the cell line MDA-MB-468 as expressing approximately $1\text{-}2 \times 10^6$ EGFR/cell, approximately 20-100-fold higher than levels expressed in other breast tumor cell lines or normal fibroblasts (Filmus et al, 1985). In this case the over-expression is based on an amplification of the receptor gene; Southern blotting indicates a 20-40-fold amplification and in situ hybridization locates the amplified domain to an abnormally-banding region on one copy of chromosome 7 (Filmus et al, 1987,a). No evidence of structural rearrangements in the EGFR gene have been detected. In addition, the properties of the over-expressed protein appear normal with respect to binding affinity, synthesis, turnover and autophosphorylation (Filmus et al, 1987,a; Kudlow et al, 1986).

GROWTH PROPERTIES OF THE MDA-MB-468 CELLS

Cell lines over-expressing the EGFR commonly can be growth inhibited in tissue culture by exposure to supra-physiological levels of EGF. The mechanism of this phenomenon has not been established, but it has been clearly related to receptor frequency; using variants selected from A431 carcinoma cells expressing different levels of receptor, Kamamoto et al, (1984) demonstrated a "threshold" requirement in terms of receptor number to allow growth inhibition to occur and in a series of squamous tumor cell lines, Kamata et al (1986) showed a direct relationship between growth inhibition and increasing receptor frequency. Despite these relationships it is of interest that over-expression of the EGFR alone does not seem to confer the ability to be growth inhibited by high levels of EGF. In circumstances where expression of EGFR is imposed on normal fibroblasts after transfection with expression vectors, clones of transformed fibroblasts are generated which express high levels of EGFR but which are not growth inhibited by supraphysiological levels of EGF (Haley et al, 1989; De Fiore et al, 1988).

In keeping with the experience with squamous carcinoma cells, MDA-MB-468 is also growth inhibited in tissue culture by high levels (10^{-7}M) of EGF (Filmus et al, 1985). Clones selected for survival under these conditions were all found to express low numbers (approximately 2×10^4/cell) of receptor. In 12 selected clones the mechanism used to overcome the EGF-growth inhibition was loss of the copy of chromosome 7 bearing the amplified allele of the EGFR. This was demonstrated both by karyology and by the fact that the amplified allele of the EGFR in MDA-MB-468 cells has a useful restriction fragment length polymorphism (Filmus et al, 1987,a). Such variants exist within the parental MDA-MB-468 cell line at a frequency of approximately 1/10^6.

6 of the 12 subclones (S1,S4,S5,S10,S11,S12) were selected for investigation of EGF-mediated growth control. In plastic-adherent tissue culture containing 10% Fetal Calf Serum, all 6 clones could be moderately stimulated in terms of growth by the addition of EGF, and supra-physiological conditions did not cause growth inhibition. When suspended in agar-containing medium, clones S1,S5,S10,S11 and S12 displayed the same phenotype as in adherent culture. However, clone S4 is dependent on the addition of exogenous EGF for growth in agar (Filmus et al, 1987,a). Similarly, when the tumorigenicity of the clones was assessed, S4 displayed a phenotype separate from the other 5 clones. Clones S1,S5,S10,S11, and S12 are all tumorigenic, forming progressively-growing tumors after subcutaneous implantation of 10^6 cells in the flank or mammary fat pad of nude mice. Clone S4 however, is non-tumorigenic under the same conditions. When the rates of tumor growth of clones S1,S5,S10,S11 and S12 were compared with parental MDA-MB-468 cells, a growth rate advantage could be detected for the parental cell line (Doubling time of 6 days vs. 10 days).

The tumor growth experiments described above suggest that amplification of the EGFR gene and subsequent over-expression of the receptor protein provides a growth advantage _in vivo_. Similar conclusions have been reached through study of the _in vivo_ growth of A431 and variant cell lines (Santon et al, 1986). These experiments provide a biological rationale for the _in vivo_ selection of tumor cell clones over-expressing the EGFR during tumor progression. Based on analysis of receptor expression in relation to tumor stage (Harris and Nicholson, 1988), over-expression of EGFR is likely a late event in tumorigenesis. Our data are consistent with that view in that the predominant class of subclones (S1,S5,S10,S11,S12) are still tumorigenic and therefore must possess other derangements of cell growth regulatory mechanisms. One such abnormality was described recently; MDA-MB-468 cells have a homozygous deletion of the recessive oncogene RB1 which confers susceptibility to retinoblastoma and possibly also to other tumor types (Lee et al, 1988).

We have been unable, as yet, to explain the different growth properties of S4 and the predominant class of subclones. There are two plausible explanations for the differential response to EGF in agar culture which are suggested by other work in the area of growth control. Firstly, the EGFR in subclone S4 might have a subtle alteration in affinity or half-life causing a decreased requirement for EGF or secondly, subclone S4 might depend less on the production of autocrine growth factors than the other clones.

TABLE 2. Comparison of EGFR Properties (A) and Transcription of Transforming Growth Factors Genes (B) in MDA-MB-468 and Subclones S4 and S5

(a)	468	S4	S5
EGFR Freq.	2×10^6	3.3×10^4	1.8×10^4
KD (M)	4.0×10^{-10}	4.8×10^{-10}	3.7×10^{-10}
$T\frac{1}{2}$(-EGF)(hrs)	21 ± 4	20 ± 5	15 ± 2
(b)			
TGF α mRNA	++	+	+
TGF β mRNA	++	+	+

As shown from the data in Table 2 neither of these possibilities seems to be operating in this case. The receptors in S4 and S5 are indistinguishable in terms of frequency, affinity or half-life. In addition the receptors of both clones do not differ from the parental cells in terms of affinity or half-life. We have also quantitated steady state levels of transcripts of the TGFα and TGFβ genes; the products of these genes have been implicated in the control of breast cancer cells (Dickson and Lippman, 1988) including MDA-MB-468 (Fernandez-Pol et al, 1987). Although expression of both genes in the subclones is less than in the parental cells, no differences between S4 and S5 could be detected. This, of course, does not rule out differences at the protein level of these potentially autocrine growth regulators since regulation of their production might be post-transcriptional under some circumstances.

On the basis of these preliminary experiments we believe that the mechanism involved in the EGF-dependency of subclone S4 likely lies in other cellular gene expression which probably is regulated by EGF specifically in anchorage-independent tissue culture. Clone S4 may therefore represent a useful source of information on gene expression necessary for anchorage-independent growth.

MECHANISMS OF EGF SIGNAL TRANSDUCTION IN MDA-MB-468

The intracellular events contributing to signal transduction from the EGFR are not totally understood. A number of events subsequent to EGF binding have been documented including phosphorylation of a number of protein substrates, receptor autophosphorylation, activation of the Na^+/H^+ exchange system resulting in cytoplasmic alkalinization, mobilization of intracellular calcium stores, stimulation of phosphatidyl inositol (PI) turnover and changes in gene expression (particularly induction of expression of c-myc and c-fos genes) (Carpenter, 1987). Of these events, only activation of the receptor kinase has been shown to be essential for signal transduction, since various mutant EGFR'S devoid of kinase activity are unable to transduce signals (Honegger et al, 1987). PI turnover is frequently activated by EGF stimulation, particularly in cells over-expressing EGFR (Wahl et al, 1988). In A431 cells it has been suggested that the linkage between EGFR and the generation of second messengers may be achieved through the fact that phospholipase C, the enzyme responsible for PI turnover, is a substrate for the tyrosine phosphorylation catalyzed by the activated receptor (Wahl et al, 1989).

The growth properties of MDA-MB-468 and subclones provided an opportunity to assess differential mechanisms of signal transduction under circumstances of negative and positive growth regulation. For example, in these cell lines, as in other cells responsive to EGF, an early consequence of EGFR activation is the transient accumulation of transcripts of the c-*myc* and c-*fos* genes. It is considered possible that the products for these genes act to regulate transcription of other genes and thus play a role in the altered transcription elicited by EGF. We found that the kinetics of the altered accumulation of c-*myc* and c-*fos* transcript was identical for all cell lines; maximum at 30' for c-*myc* mRNA and 15' for c-*fos* mRNA. In addition, we found that the magnitude of the increased accumulation was similar in the parental cell line and subclones, despite the fact that EGF is acting as growth inhibitor and stimulator respectively (Filmus, et al, 1987). Therefore, the transient elevation in level of transcript and subsequent protein expression from the c-*myc* and c-*fos* genes do not seem to play a role in the selectivity of the action of EGF.

Further evidence which dissociates c-*myc* and c-*fos* transcription from EGF-induced growth inhibition or mitogenesis came from experiments designed to assess the sensitivity of MDA-MB-468 and subclone S4 to pertussis toxin. We initiated these experiments since many hormone receptor systems are linked to the generation of second messengers by the interaction of G-proteins. This family of proteins has the property of linking receptor occupancy to activation of adenyl cyclase, phospholipase C or ion channels (Milligan, 1988). A feature of certain G protein α-subunits is a sensitivity to ADP-ribosylation by pertussis toxin (P.T.); demonstration of an attenuation of hormone effect by P.T. has therefore been used to define the role of G-protein intermediates in the signal transduction of various hormones (Milligan, 1988).

We demonstrated that P.T. could block both EGF-induced growth inhibition in MDA-MB-468 in anchorage-dependent or independent culture and the EGF-dependent growth of clone S4 in anchorage-independent conditions (Church and Buick, 1988). This indicated the possibility of a G-protein intermediate in EGF signal transduction. A role for such intermediates has also been suggested by the demonstration of EGF-mediated phosphorylation of a G-protein β subunit in human placental membranes (Valentine-

Braun et al, 1986) and by the P.T. sensitivity of proliferative responses of hepatocytes to EGF (Johnson et al, 1986). The molecular characteristics of the G-protein intermediate in MDA-MB-468 cells has not been elucidated. Under the conditions of the experiments, P.T. catalyzed the transfer of ADP-ribose from NAD^+ to an intrinsic membrane protein(s) of approximately 40 Kd. Therefore, it seems likely that the target for the ADP-ribosylation of the P.T. is an α-subunit of one of the trimeric G-protein family. Since cAMP is not affected under these conditions, the effective G protein is not the G_i protein coupling to adenyl cyclase. It is of interest that the G-protein-mediated pathway of EGF signal transduction may have some specificity for this particular cell line since we have shown that EGF growth inhibition of A431 cells is not P.T. sensitive.

Although P.T. is able to block the proliferative changes associated with EGF exposure in MDA-MB-468 and subclone S4, these same conditions did not block the increased accumulation of c-*myc* and c-*fos* transcripts (Church and Buick, 1988). This implies either; (a) that the P.T. sensitive event exists on a separate pathway of transduction from that causing the transcription of c-*myc* and c-*fos* genes or (b) that the P.T. sensitive event exists on the same pathway from that causing c-*myc* and c-*fos* transcription, but at a distal position.

These possibilities are shown schematically in Fig. 1. Given the traditional role of G-protein intermediates in coupling activated membrane receptors to second messenger systems, the multiple pathway model (A) seems to be the most probable. We are currently working to assign early biochemical events in EGF signal transduction to P.T. sensitive or insensitive classes of transducing pathways.

One early consequence of EGF binding is cytoplasmic alkalinization caused by the activation of the Na^+/H^+ exchange antiport. Such early changes also occur in both MDA-MB-468 and subclone S4 in response to EGF despite the opposite proliferative response of the two cell lines (Church et al, 1989). We have used two lines of evidence to rule out an obligatory role for such activation in the EGF signal transduction process.

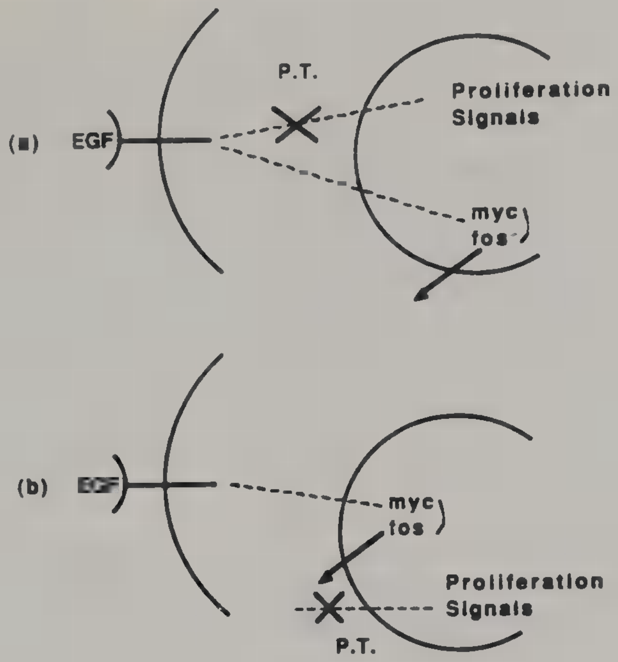

Figure 1. Schematic representation of two possible roles for P.T.-sensitive step in EGF signal transduction. Membrane bound EGFR (left) and nucleus (right). (a) Dual pathways (b) single pathway.

Blocking Na^+/H^+ exchange in both cell lines with amiloride analogues or by manipulating extracellular pH failed to block the ability of EGF to inhibit growth of MDA-MB-468 or stimulate growth of subclone S4. Furthermore, activation of Na^+/H^+ exchange is unable to alter accumulation of c-myc and c-fos transcripts in the absence of EGF. The Na^+/H^+ exchange system therefore seems to be activated by a separate EGF-mediated transduction system distinct from those responsible for growth inhibition/stimulation or for c-myc/c-fos transcription. A summary of the characteristics of EGF signal transduction in MDA-MB-468 and subclone S4 is shown in Table 3.

TABLE 3. Characteristics of EGF Signal Transduction in MDA-MB-468 and Subclones S4

A Accumulation of transcripts of c-*myc* and c-*fos* genes

(1) occurs in MDA-468 and MDA-468-S4 with identical Kinetics after EGF binding. (max. 15' for c-*fos* and 30' for c-*myc*)

(2) not affected by pertussis toxin under conditions which abrogate proliferative effects of EGF

(3) not induced by activation of Na^+/H^+ in absence of EGF

B Activation of Na^+/H^+ Exchange

(1) Amiloride analogues (EPPA and EPA) block EGF-induced cytoplasmic alkalinization, but do not affect EGF-induce growth inhibition of MDA 468 or growth stimulation of MDA-468.

(2) Low pH media abrogate EGF-induced cytoplasmic alkalinization but do not affect growth inhibition or stimulation

(3) Activation of Na^+/H^+ exchange is not able to induce c-*myc* and c-*fos* transcription.

CONCLUSION

The breast cancer cell line MDA-MB-468 has provided a useful model of the abnormal growth regulation elicited by over-expression of the EGFR. It is possible that a high proportion of estrogen-receptor negative breast tumors have acquired an *in vivo* growth advantage through this mechanism. Furthermore, the characteristics of MDA-MB-468 and variant subclones have allowed a dissection of the biochemical events leading to EGF signal transduction. In particular, these cell lines have a reliance on a novel G-protein-dependent pathway to couple the activated EGFR to signal processing.

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INHIBITORS OF BOMBESIN-STIMULATED INTRACELLULAR SIGNALS: INTERRUPTION OF AN AUTOCRINE PATHWAY AS A THERAPEUTIC STRATEGY IN SMALL CELL LUNG CARCINOMA

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INTRODUCTION

Bombesin is a tetradecapeptide originally isolated from the skin of the amphibian Bombina bombina(Erspamer and Melchiorri, 1973). It has important pharmacologic effects in mammals as a pituitary, gut, and pancreatic(exocrine and endocrine) secretogogue. This prompted investigations to define a mammalian bombesin-like peptide. McDonald et al.(1979) described a 27 amino acid peptide obtained from non-antral porcine stomach which also possessed a prominent capacity to increase gastric acidity through release of gastrin. This peptide demonstrated identity in nine of its ten carboxyl-terminal amino acids to the amphibian bombesin, and was called gastrin-releasing peptide(GRP). GRP represents at least one source of mammalian bombesin-like immunoreactivity. Wharton et al. (1978) demonstrated bombesin-related immunoreactivity in fetal lung. The peptide was localized to pulmonary endocrine cells, which possess dense-core granules(Bensch et al., 1965) as evidence of a secretory function. This immunoreactivity is widely distributed in the mammalian nervous system, as well as gut and lung(Panula, 1986).

Small cell lung carcinoma(SCLC) comprises 25% of pulmonary neoplasms. It represents a clinical subtype of lung carcinoma with several features suggestive of a relationship to pulmonary endocrine cells. For example,it is associated infrequently with the appearance of clinically significant syndromes due to the elaboration of peptide homones such as

ACTH and vasopressin. In addition, neuronal markers such as creatine kinase B type, neuron-specific enolase, as well as dense core granules on electron microscopy are commonly observed. Moody et al.(1981) demonstrated that SCLC as opposed to other histologic types of lung carcinoma have prominent expression of bombesin-like immunoreactivity. Spindel et al. (1984) derived a cDNA clone for human proGRP, which demonstrated that GRP was cleaved from a larger prohormone precursor. Using a cDNA clone for human proGRP derived by Spindel et al.(1984), Sausville et al.(1986) showed that the bombesin-like immunoreactivity found in SCLC correlated with expression of proGRP mRNA, a conclusion consistent with the studies of Yamaguchi et al. (1983) using region-specific antisera for different portions of mammalian GRP. In addition, alternative splicing of the proGRP precursor mRNA was demonstrated, allowing production of three forms of GRP prohormone, differing in the predicted amino acid sequence of the carboxyl-terminal, non-GRP portion of the prohormone.

Rozengurt and Sinnett-Smith(1983) demonstrated that in Swiss mouse 3T3 cells bombesin was mitogenic, and was synergistic with insulin in this capacity. Heslop et al.(1986) showed that a very early reponse to bombesin agonists in Swiss 3T3 cells results in hydrolysis of membrane phosphotidylinositol(PI) precursors, with initial production of inositol(1,4,5) trisphosphate(I(1,4,5)P_3), followed by more delayed production of inositol(1,3,4)trisphosphate. Mendoza et al. (1986), Rozengurt and Sinnett-Smith(1987) and Takuwa et al. (1987) have also demonstrated that bombesin-agonist stimulation of 3T3 cells results in Ca^{2+} mobilization from internal as well as external sources, coincident with protein kinase C activation, and activation of c-*fos* and c-*myc* transcription.

The fact that bombesin agonists could act as mitogens for the murine fibroblast cell line raised the possibility that in SCLC an autocrine circuit as defined originally by Sporn and Todaro(1980) could be functioning to promote growth. Subsequent experiments have demonstrated that under conditions of clonogenic growth in soft agar(Carney et al., 1987) or in tumor implants in athymic mice(Alexander et al., 1988), stimulation of SCLC cell growth by bombesin agonists may be seen. In addition, generation of an antibody against the carboxyl-terminal domain shared by bombesin and GRP also inhibited the growth of SCLC explants in athymic mice(Cuttitta et al., 1985). Thus, it became of interest to inquire if interruption of bombesin-mediated signal transductions could also allow an approach to the development of therapeutic strategies useful in SCLC.

MATERIALS AND METHODS

Cell lines

Human SCLC cell lines were developed at the NCI-Navy Medical Oncology Branch(Carney et al., 1985), and were routinely grown in RPMI-1640 medium supplemented with 30 nM sodium selenite, 5ug/ml insulin, and 1 ug/ml transferrin(R-SIT). Labelling of phosphatidylinositol precursors was accomplished by growth of the cells in 4 - 5 uCi/ml of *myo*-[^{3}H]inositol(16.3 Ci/mmol; Amersham) in RPMI-1640 formulated without unlabelled inositol, also containing selenite, insulin, and transferrin.

Biochemical assays

Phosphatidylinositol turnover was measured using HPLC separations of water-soluble inositol metabolites as described in by Trepel et al. (1988a). Assays of alterations in intracellular free [Ca^{2+}]([Ca^{2+}]$_i$) were conducted as described by Heikkila et al.(1987) using the quin2 method of Tsien et al.(1982). Analysis of c-,N-, L-*myc* oncogene and proGRP gene expression was accomplished using Northern blot techniques described in detail elsewhere(Sausville et al., 1986; Nau et al., 1986; Kaye et al., 1988)

RESULTS AND DISCUSSION

Table 1 demonstrates that SCLC cell lines which express readily detectable levels of GRP mRNA are distinctive in two respects. First, these cell lines in general are also biochemically responsive to bombesin agonists, with increases in [Ca^{2+}]$_i$ apparent following addition of the ligand [Tyr^4] bombesin. Second, best responsiveness biochemically to GRP occurs in SCLC lines which express L-*myc* and lack constittutive high level c-*myc* expression. N-*myc* expression also correlates with lack of responsiveness to the ligand. It is also of interest that L-*myc*-expressing SCLC are those with easily demonstrable endogenous GRP expression. These data correlate with the observation that L-*myc* and *ras* co-

TABLE 1

CORRELATION OF BOMBESIN RESPONSIVENESS WITH MYC/GRP GENE EXPRESSION

CELL LINE	% INCREASE $[Ca^{2+}]_i$	L-*MYC*	N-*MYC*	C-*MYC*	GRP
NCI-H209	57	+	-	-	+
NCI-H298	34	+	-	-	N.D.
NCI-H345	55	+	-	-	+
NCI-H378	12	+	-	-	+/-
NCI-H510	71	+	-	-	+
NCI-H82	0	+/-	-	+	-
NCI-N417	0	-	-	+	-
NCI-H69	0	-	+	-	N.D.
NCI-592	0	-	+	-	-

transfection of primary rat embryo fibroblasts does transform the cells, but at a considerably lower frequency than c-*myc*(Birrer et al., 1988). Thus, a GRP-responsive autocrine circuit may be seen as most relevant to SCLC cell lines which utilize a *myc*-gene family member of relatively low transforming and perhaps proliferation-inducing potential.

There are at least three potential explanations for this behavior. First, the non-responsive cell lines may simply lack receptor for bombesin congeners. Second, N-*myc* or c-*myc* overexpression may reduce the transduction of a signal from the bombesin receptor to intracellular effectors. Third, N-*myc* or c-*myc* overexpression may not interact with the bombesin receptor in a biochemical sense, but convey upon those cells a growth advantage irrespective of their expression of the bombesin receptor. Cellular utilization of an autocrine circuit involving GRP would have no selective advantage in this cell population, which might come to predominate in a given cell line. The last possibility is particularly intriguing, as it is expected that events in the SCLC-tumor bearing host might resemble this circumstance. In addition, it allows for the possible importance of infrequent(in the population) but clonogenic cells still responsive to bombesin. This is of relevance because previous experiments have suggested that some SCLC cell lines not responsive in a biochemical sense to bombesin or GRP may still be stimulated at low frequency in clonogenic growth by the peptide (Carney et al., 1987; Cuttitta et al., 1985).

The data in Table 1 are therefore consistent with the operation of an autocrine mechanism most prominently in cell lines which do not have high level c- or N-*myc* expression. If an autocrine circuit is potentially useful for exploitation in the development of therapeutic approaches to SCLC, a clear delineation of the biochemical pathways activated by bombesin congeners in these cells is of importance. Interruption of these pathways could be a mechanism to decrease cell growth in cells lines which utilize this autocrine circuit.

Fig. 1 demonstrates that increases in $[Ca^{2+}]_i$ evoked by bombesin agonists in SCLC conform to a structure-activity relationship described for bombesin congeners in other systems (Broccardo et al., 1975). Specifically, $[Tyr^4]$bombesin, $[Leu^4]$bombesin, GRP, GRP$^{20-27}$, and ranatensin all increase $[Ca^{2+}]_i$ equipotently, whereas the amino-terminal fragment GRP$^{1-16}$, not possessing the homologous, conserved domain shared by the amphibian bombesin and mammalian GRP shows no such activity. GRP$^{1-16}$ also does not interfere with added bombesin. Thus, the bombesin agonist-induced increase in $[Ca^{2+}]_i$ is specific and, for example, not evoked by the tachykinin physalemin.

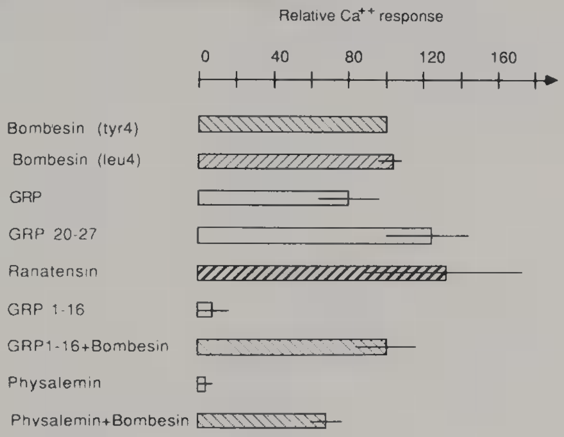

Fig. 1. STRUCTURE ACTIVITY RELATIONSHIP OF BOMBESIN AGONISTS TO INCREASE $[Ca^{2+}]_i$ IN SCLC
$[Ca^{2+}]_i$ was measured by the quin2 fluorescence after addition of 100 nM of the indicated agonist. The % of basal $[Ca^{2+}]_i$ which increased after peptide addition is shown.

As shown in detail elsewhere, bombesin-stimulated increases in $[Ca^{2+}]_i$ are transient in Swiss mouse 3T3 cells and SCLC. Specifically, the peak increase occurs within seconds, and returns toward basal concentrations after 3-5 min of stimulation(Mendoza et al., 1986; Takuwa et al., 1987; Heikkila et al., 1987). The exact source of this Ca^{2+} is not entirely clear. A major component derives from intracellular sources, as there are significant increases in $[Ca^{2+}]_i$ even in the presence of EGTA. However the magnitude of the increase is dampened, and its duration is shortened. In addition, evidence exists for stimulation by bombesin of $^{45}Ca^{2+}$ entry into 3T3 cells(Takuwa et al., 1987). Thus, the possibility of a bombesin-stimulated Ca^{2+} entry channel or pathway from the extracellular space exists, as has been suggested for a number of Ca^{2+}-mobilizing ligands(Meldolesi and Pozzan, 1987).

A schematic outline of the events leading to the release of Ca^{2+} from intracellular stores is shown in Fig. 2. A hormone sensitive phospholipase-C has been shown in a variety of systems(Berridge, 1987) to result in the rapid generation of $(1,4,5)P_3$ very shortly after ligand stimulation, resulting from hydrolysis of phosphatidylinositol(4,5)bisphosphate (PIP_2). Successive dephosphorylations result in inositol-(1,4)bisphosphate($I(1,4)P_2$) and the inositol monophosphate I-4-P. While theoretically I-1-P can also result from dephosphorylation of $I(1,4)P_2$, recent experiments in GH_3 pituitary cells have shown that I-1-P appears with a delay

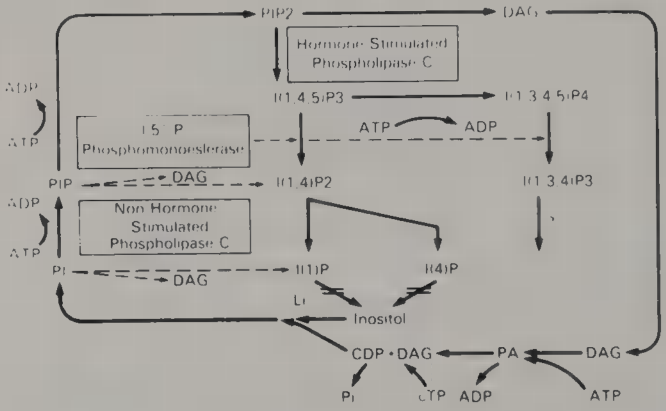

FIG. 2. SCHEMATIC OUTLINE OF PHOSPHATIDYLINOSITOL METABOLISM

in comparison to I-4-P(Dean and Moyer, 1987). It can be appreciated, however, that action of phospholipase(s)-C on the membrane lipids phosphatidylinositol-4-phosphate(PIP) and phosphatidylinositol(PI) can also give rise to I(1,4)P_2 and I-1-P, respectively. While these latter two metabolites cannot mobilize Ca^{2+}, hydrolysis of PIP and PI leading to their formation can produce diacylglycerol(DAG) and thus contribute to the activation of protein kinase C(Berridge, 1987). Therefore, increased PI turnover in response to ligand can be thought of as potentially reflecting at least two types of hormonal activation of phospholipase: in one case proceeding through PIP_2 hydrolysis coupled to receptor occupation to yield I(1,4,5)P_3, and in the second case, through stimulated hydrolysis of PIP and PI which may be indirectly linked occupation of receptor by ligand. Since phospholipase(s)-C are activated by Ca^{2+}in many important cases (Uhing et al., 1986; Ryu et al., 1987; Nakanishi et al., 1985, Bennett and Crooke, 1987, Rebecchi and Rosen, 1987) , it is possible that rapid hormone-induced increases in $[Ca^{2+}]_i$ may actually increase the intrinsic activity of phospholipases in a way not coupled directly to ligand receptor activation.

Also shown in Fig. 2 is the inhibition by Li^+ of the hydrolysis of I-4-P and I-1-P which would yield free inositol. This permits experiments conducted in the presence of Li^+ to observe PI metabolism after "trapping" labelled metabolites as monophosphates.

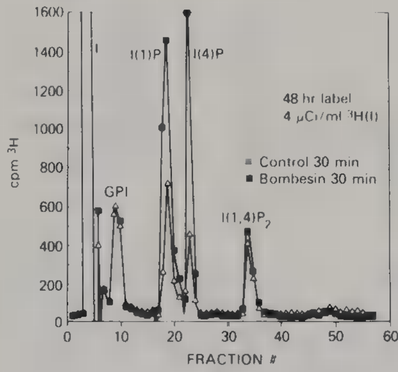

FIG. 3. STIMULATION OF PI TURNOVER IN NCI-H345 BY [TYR^4]-BOMBESIN. Cells labelled with *myo*^{3}H-inositol were treated with 600 nM [Tyr^4]bombesin for 30 min, in the presence of 0.01 M LiCl,and acid-soluble label analyzed by HPLC(Trepel et al., 1988a).

Fig. 3 demonstrates that treatment of NCI-H345 SCLC cell line with [Tyr^4]bombesin in the presence of Li$^+$ increases I-4-P and I-1-P after 30 minutes of stimulation. No significant increases in I(1,4,5)P_3 are seen at that time. but prior studies(Trepel et al., 1988a had shown in these cells that I(1,4,5)P_3 reached maximal increases after ~8 sec, and was declining by 30 sec of stimulation.

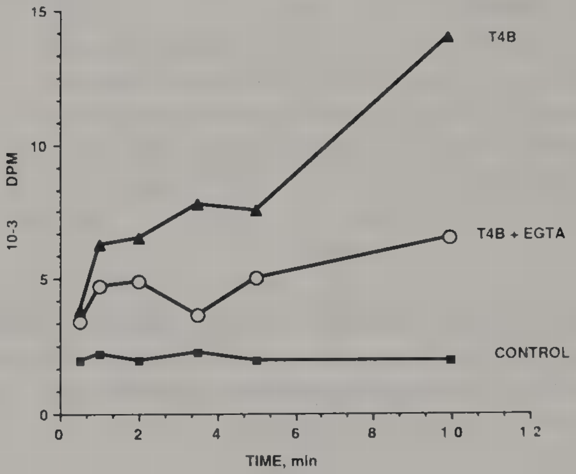

FIG. 4. EFFECT OF EXTERNAL EGTA ON BOMBESIN AGONIST-INDUCED INCREASE IN I-4-P. NCI-H345 was prelabelled with *myo*[^{3}H]-inositol and cells were stimulated in the presence of LiCl with 200 nM [Tyr^4]bombesin with or without 8mM EGTA added 15 min before stimulation with ligand. "Control" cells received neither ligand nor EGTA. After stimulation for the indicated period, acid-soluble extracts were examined by HPLC(Trepel et al., 1988a) for label in I-4-P.

Fig. 4 examines the effect of external EGTA on the generation of I-4-P. It can be seen that EGTA in the external medium(added immediately prior to ligand addition inhibits the production of I-4-P in response to bombesin agonist addition. However, it will be noted that increases in I-4-P at late times after stimulation(e.g., 10 min) are more clearly inhibited(~50%) than at very early times after stimulation (e.g., ~25-30% at 1 min). This data raises the possibility that two processes are contributing to the increase in I-4-P observed after treatment withbombesin agonists: a process sensitive to EGTA that is quantitatively very significant at relatively late times after agonist stimulation, and a process active at early times after stimulation that is more refractory to EGTA and therefore by inference less influenced by the presence of Ca^{2+} in the extracellular space.

A second potential way of defining ligand-dependent pathways leading to PI turnover is to examine the effects of bombesin receptor antagonists. Coy et al. (1988) designed a reduced peptide bond receptor antagonist [Leu^{13}-Ψ-CH$_2$NH-Leu^{14}]bombesin with a K$_i$ for inhibition of ~1 nM of ^{125}I-[Tyr^4]bombesin binding of approximately 80 nM. In the experiment shown in Fig. 5, addition of 500 nM of this antagonist potently decreased the elaboration of I(1,4,5)P$_3$ observed eight seconds after 100 nM [Tyr^4]bombesin addition. In contrast, bombesin agonist-induced elaboration of I-4-P observed after 30 min of stimulation was considerably less sensitive to the antagonist.

Previous studies from our laboratory (Trepel et al., 1988b) have defined that the antagonist's IC$_{50}$ for [Tyr^4]bombesin-induced I(1,4,5)P$_3$ production in SCLC at eight seconds is ~50 nM vs 100 nM agonist, and ~5 nM vs a submaximal concentration of 10 nM agonist. In addition, [Leu^{13}-Ψ-CH$_2$NHLeu^{14}] -bombesin displayed no partial agonist activity in this assay. Taken together with the results in Fig. 5, it

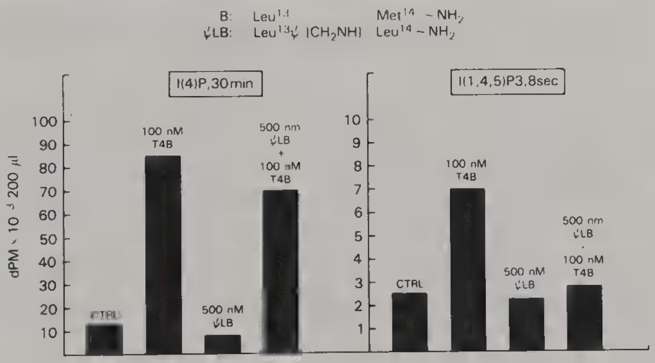

FIG. 5. DIFFERENTIAL INHIBITION OF INOSITOL PHOSPHATE ISOMERS BY [LEU^{13}-Ψ-CH$_2$NHLEU^{14}]BOMBESIN. NCI-H345 SCLC were labelled with *myo*-[^{3}H]inositol, and stimulated with 100nM [Tyr^4]bombesin(T4B) in the presence of 0.01 M LiCl with or without the prior addition(5 min) of 500 nM [Leu^{13}-Ψ -CH$_2$NHLeu^{14}]bombesin (Ψ LB). Acid-soluble label was examined by HPLC(Trepel et al., 1988a), after incubation for eight sec(I(1,4,5)P$_3$) or after 30 min(I-4-P).

might be predicted that the antagonist should be markedly less potent in the inhibition of I-4-P production. This prediction is borne out in Fig. 6., where it can be seen that [Leu^{13}-Ψ-$\mathrm{CH_2NHLeu}^{14}$]bombesin has an IC_{50} of approximately 10-15 uM vs 100 nM [Tyr^4]bombesin for inhibition of I-1-P and I-4-P elaboration after thirty minutes of hormone stimulation.

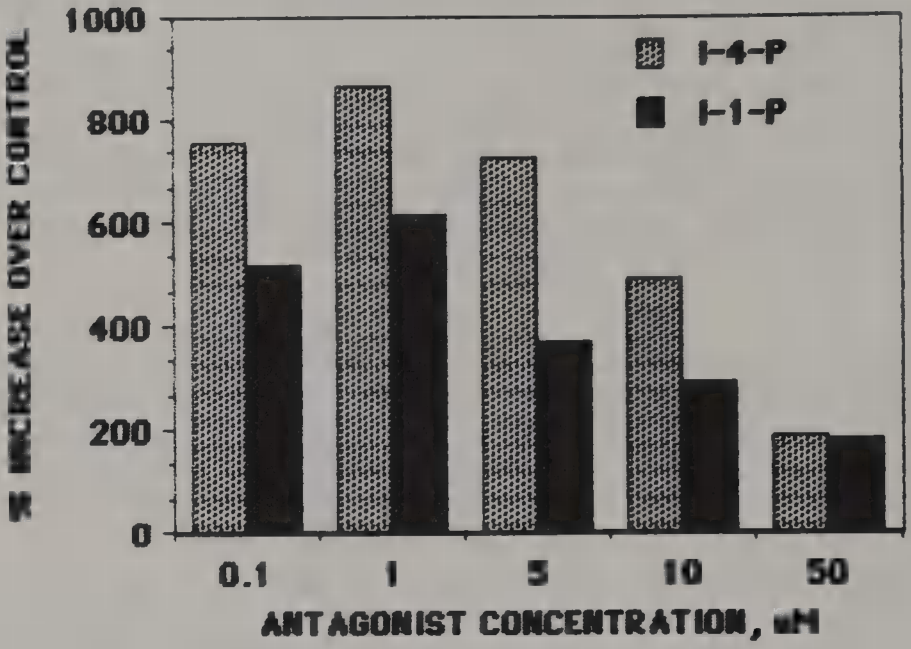

FIG. 6. INHIBITION OF INOSITOL PHOSPHATE PRODUCTION BY [LEU^{13}-Ψ -$\mathrm{CH_2NHLEU}^{14}$]BOMBESIN. [^{3}H]inositol-labelled NCI-H345 were treated with the indicated concentrations of antagonist 5 min prior to addition of 100 nM [Tyr^4]-bombesin in the presence of 0.01 M LiCl. Following incubation for 30 min, labelled I-4-P was examined by HPLC(Trepel et al., 1988a).

Additional important features in the action of this bombesin receptor antagonist are that its IC_{50} for [Tyr^4]bombesin-induced Ca^{2+} mobilization is approximately 5-10 uM vs 100 nM of agonist, and that it inhibits the clonnogenic growth of SCLC in soft agarose(Trepel et al., 1988b).

It is intriguing to speculate that the sustained PI turnover observed in the 30 minute incubation with bombesin agonists, which is inefficiently inhibited by [Leu^{13}-ψ-$\mathrm{CH_2NHLeu}^{14}$]bombesin is related to the PI turnover which requires Ca^{2+} entry into cells. This data recalls observations of (Imai and Gershengorn, 1986) that PIP$_2$ hydrolysis is transient in pituitary cells stimulated with TRH, yet PI turnover sustained. This behavior is consistent with a model in SCLC wherein the initial increase in Ca^{2+} may have a persistant effect in activating a phospholipase-C which is itself not directly coupled in a molecular sense to occupation by ligand of receptor

Alternative models to account for the behavior of [Leu^{13}-ψ-CH$_2$NHLeu^{14}] bombesin may, however, be considered. A trivial explanation of these data could be that the stability of the antagonist is less than that of [Tyr^4]bombesin in these systems. It is also possible that the phospholipase directly coupled to receptor responsible for hydrolysis of PIP$_2$ differs from a hypothetical phospholipase also coupled to receptor but which hydrolyzes PIP or PI. If this were true, it is conceivable that the antagonist could have differential capacity to inhibit the coupling of one receptor to these two distinct transduction mechanisms. In an analogous fashion the coupling of the receptor to a putative receptor-operated Ca^{2+} channel may be less efficiently inhibited by the antagonist than is the coupling to the PIP$_2$-hydolyzing phospholipase-C.

Taken together with our previously published studies using [Leu^{13}-ψ-CH$_2$NHLeu^{14}]bombesin(Trepel et al., 1988b), these experiments are therefore consistent with the possibility that interruption of a putative autocrine stimulus is a reasonable approach toward designing inhibitors of SCLC growth. Since conventional therapy, including chemotherapy with or without radiation therapy can result in substantial regression of this tumor, yet the median survival for all patients with optimal treatment varies between eight months (extensive stage) or 18 months (limited stage), use of a bombesin antagonist as an adjuvant to cytoreductive therapy could have wide applicability. In this regard, the development of bombesin receptor antagonists of even more favorable binding characteristics and stability in vivo than possessed by [Leu^{13}-ψ-CH$_2$NHLeu^{14}]bombesin will be of great interest in designing needed clinical trials. The results presented here emphasize that inhibition of growth factor-dependent PI turnover may proceed with different degrees of effectiveness, depending on whether the inhibitor under consideration can very efficiently inhibit the transient generation of I(1,4,5)P$_3$ alone, or whether it also inhibits sustained PI turnover as well. In this respect, growth factor receptor antagonists can provide important information concerning pathways of cellular activation by the growth factor in question.

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HER-2/*neu* ONCOGENE AMPLIFICATION AND EXPRESSION IN BREAST AND OVARIAN CANCERS

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INTRODUCTION

There is considerable circumstantial evidence suggesting that cellular oncogenes play a role in the pathogenesis of malignancies. Alterations in the structure, copy number or expression of some oncogenes may be important factors in the initiation and/or progression of some human malignant neoplasms. N-myc amplification of adrenal neuroblastomas in children is correlated with advanced clinical stage and shorter overall survival (Seeger et al, 1985). Preliminary results suggest that oncogene amplification and/or expression may be clinically important in managing patients with lung cancer. Oncogenes of the myc family (c-myc, L-myc, M-myc) are often amplified in small cell carcinomas of the lung (Little et al, 1983; Nau et al, 1986; Wong et al, 1986). The epidermal growth factor receptor (c-erbB) is amplified and overexpressed in approximately 10% of non-small cell lung carcinomas (Koga et al, 1989). Recently, amplification of the HER-2/*neu* oncogene has been associated with a shorter disease-free interval and shorter overall survival in women with breast cancer (Slamon et al, 1987; 1989a,b). In addition, HER-2/*neu* amplification and overexpression has been associated with a shorter survival in women with ovarian cancer (Slamon et al, 1989b).

The *neu* oncogene was first isolated from DNA extracted from ethylnitrosourea-induced adrenal neuroglioblastomas of neonatal rats (Shih et al, 1981). The oncogene was identified by its ability to function as a dominant transforming gene in NIH 3T3 cell transfection assays. Since this gene was derived from neuroglioblastomas it was referred to as *neu*

(Schechter et al, 1984). Subsequently, three different groups independently identified the human homolog of this gene (Coussens et al, 1985; Semba et al, 1985; King, Kraus and Aaronson, 1985) and, because of homology with the human epidermal growth factor receptor (HER) and c-erb-B proto-oncogene, it was referred to as HER-2 (Coussens et al, 1985) or c-erbB-2 (Semba et al, 1985).

Considerable interest in the HER-2/*neu* oncogene was aroused by the observation that amplification of this gene was correlated with both a shorter disease free interval and a shorter overall survival in women with node positive breast cancer (Slamon et al, 1987). The initial study reported Southern blot analyses of 189 breast cancers. In this cohort amplification was correlated with the number of involved axillary lymph nodes. However, only 86 of the reported cases, all with involved lymph nodes, had clinical followup information available and in this group those cases with more than 5-fold amplification of the HER-2/*neu* oncogene had a shorter disease-free and overall survival than those without amplification of the gene. In subsequent studies Southern hybridization confirmed that HER-2/*neu* was amplified in breast cancers but the exact percentage of cases showing amplification was disputed (Ali et al, 1988; Slamon and Clark, 1988). In addition other studies, also performed on small series of cases, yielded conflicting results. Using Southern blot analysis some found a correlation between HER-2/*neu* gene amplification and outcome (Varley et al, 1987) and others did not (Zhou, Ahuja and Cline, 1989). Several groups have used antibodies recognizing p185$^{\mathrm{HER\text{-}2}/neu}$ for immunohistochemical detection of the protein product in tissue sections. With one exception (Venter et al, 1987), these studies have used archival paraffin embedded tissue sections. Immunohistochemical staining has likewise been reported to correlate (Wright et al, 1989), not to correlate (Barnes et al, 1988; Gusterson et al, 1988; Machin et al, 1988), or to be of borderline significance (Van de Vijver et al, 1988) with respect to clinical outcome. However, most of these studies suffer from the relatively small number of cases analyzed and from evaluating only one aspect of the gene (DNA, RNA or protein). We decided to expand both the number of breast cancers evaluated and the number of analyses performed on each breast cancer.

AMPLIFICATION OF HER-2/*neu* IN A LARGE SERIES OF BREAST CANCERS.

A total of 668 breast cancers, 526 with clinical followup information, were collected for these analyses (Slamon et al, 1989a,b). HER-2/*neu* gene amplification was determined in all cases by Southern blot analysis of extracted DNA. Comparison of HER-2/*neu* hybridization signals obtained in the DNA blots with myeloperoxidase gene hybridization signals obtained after the blots were stripped and reprobed demonstrated that 27% of the breast cancers had amplification of HER-2/*neu* gene (Slamon et al, 1989a,b). 345 women had involved axillary lymph nodes and significant clinical followup information, median followup of 57 months (60 months for those still alive). 101 of these 345 breast cancers had HER-2/*neu* gene amplification and this amplification correlated with both the disease free interval and the overall survival (Table 1). Tumor size was a stronger predictor of clinical outcome than HER-2/*neu* gene amplification on univariate analysis but lost its significance on multivariate analysis indicating that the predictive value of tumor size was not independent of nodal status. Multivariate analysis showed HER-2/*neu* amplification to be an independent predictor of both time to relapse ($p < 0.006$) and overall survival ($p < 0.045$). Amplification of HER-2/*neu* in tumor tissue was a stronger predictor of clinical outcome than other known prognostic factors with the exception of the patient's lymph node status (Table 1).

TABLE 1. Comparison of Prognostic Factors in 345 Node-Positive Breast Cancer Patients by Univariate and Multivariate Statistical Analyses.

	Disease free interval		Overall survival	
	P Value, Univariate	P Value, Multivariate	P Value, Univariate	P Value, Multivariate
Nodes	<0.0001	<0.0001	<0.0001	<0.0001
HER-2/*neu*	0.01	0.006	0.041	0.045
ER	0.235	0.60	0.091	0.157
PgR	0.045	0.07	0.20	0.24
Size	0.003	0.15	0.006	0.16
Age	0.92	0.96	0.20	0.11

Statistical analyses were performed by the X^2 test and by Cox's partially nonparametric regression analysis to evaluate the predictive power of various combinations of prognostic factors. Nodes = number of involved axillary lymph nodes; HER-2/*neu* = oncogene amplification; ER = estrogen receptor status; PgR = progesterone receptor status; Size = maximum diameter of the primary neoplasm; Age = patient age at the time of diagnosis. (reproduced from Science 244: 707-712, 1989)

DNA from 181 node-negative breast cancers was also evaluated. Forty-five of these (25%) had amplification of the HER-2/*neu* oncogene. These patients had a median followup of 59 months (62 months for those still alive) but statistical analysis failed to show a significant relationship between disease outcome and oncogene amplification.

COMPREHENSIVE ANALYSIS OF HER-2/*neu* IN BREAST CANCER.

Although HER-2/*neu* appears to be an interesting marker gene of more aggressive-behaving breast cancers, it is not likely to play a pathogenetic role in this behavior unless the gene is also expressed in these tumors. With this consideration in mind we performed a comprehensive study of some of these breast cancers in order to compare oncogene amplification with expression. Sufficient tumor tissue was available from 187 breast cancers to permit multiple analyses of the same specimen. This group included both node-negative and node-positive breast cancers and primary lesions of various sizes. We had previously obtained a cDNA clone from a human breast cancer which corresponded to the entire HER-2/*neu* coding region (Slamon et al, 1989a,b). A synthetic peptide corresponding to the carboxy terminal 14 amino acids was used to generate an antiserum which specifically recognized HER-2/*neu* protein product (Slamon et al, 1989a). The availability of these probes and the breast cancers permitted us to analyze both the gene and its products (RNA and protein) in these tissues. DNA from these cases was characterized by Southern blot analysis; the RNA by Northern blot analysis; and the protein by both Western immunoblot analysis and immunohistochemistry. Each of the assays was performed blinded to clinical followup as well as to each other.

After the analyses were completed and the results were unblinded, it was found that 51 of the 187 breast cancers had amplification of HER-2/*neu* gene (27%) and each of these cases showed overexpression of HER-2/*neu* gene products relative to the findings in cases without amplification of the gene (Figure 1). The breast cancers with no amplification of the HER-2/*neu* gene (63% of the cases) had low levels of HER-2/*neu* messenger RNA by Northern analysis, small amounts of protein product by Western analysis and little membrane staining by immunohistochemistry (Figure 1, row A). Those breast cancers with 2- to 5-fold amplification of the HER-2/*neu* gene had slightly increased levels of HER-2/*neu* message by Northern analysis, and slightly increased levels of protein product by both Western analysis and immunohistochemistry (Figure 1, center row, labeled B). Breast cancers with 5- to 20-fold and >20-fold amplification of the gene had progressively higher levels of HER-2/*neu* gene overexpression of both the messenger RNA and the protein product (Figure 1, rows C and D, respectively).

From this data it is clear that HER-2/*neu* proto-oncogene is not only amplified but is also overexpressed in breast cancer. In addition, the amount of overexpression was approximately proportional to the amplification level. However, there were 18 cases (10%) which were characterized as single copy with respect to the HER-2/*neu* gene but, nevertheless, had overexpression of HER-2/*neu* messenger RNA and

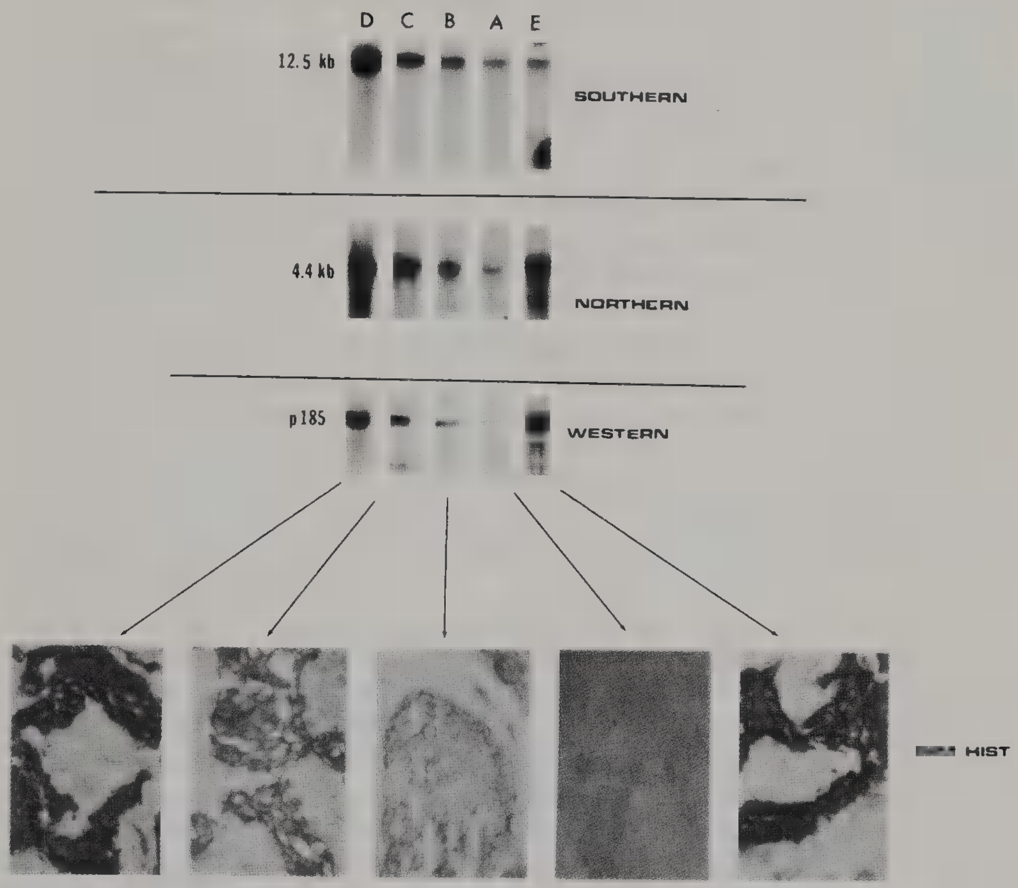

Figure 1. Examples of HER-2/*neu* gene amplification and expression analyzed by Southern, Northern, Western and immunohistochemical analyses. The location of the 12.5 kb DNA fragment which contains the HER-2/*neu* gene in EcoRI cut DNA is labeled on the ”SOUTHERN” panel. The 4.4 kb messenger RNA coding for HER-2/*neu* is indicated in the panel containing the ”NORTHERN” blots. The position of the HER-2/*neu* protein product is indicated as p185 in the ”WESTERN” panel. Across the top of the figure the various rows are labeled A, B, C, D, E to identify the rows illustrating single copy low expressors (A), 2- to 5-fold amplified overexpressors (B), 5- to 20-fold overexpressors (C), >20-fold amplified overexpressors (D), and single copy overexpressors (E) as described in the text. (reproduced from Science 244: 707-712, copyright 1989 by the AAAS.)

protein product (Figure 1, row E) suggesting that these cases may have alterations in the promoter or enhancer elements of the gene. These cases could also represent low end amplified cases (2- to 5-fold amplification of the HER-2/*neu* gene) where Southern blot analysis has underestimated the gene copy level due to dilution of carcinoma cell DNA by normal stromal cell DNA which is also present in the tumor.

Among the 51 cases with amplification of HER-2/*neu*, all showed overexpression of the gene product by two of the three measures of expression. Ninety percent (46 cases) showed complete concordance with respect to overexpression among all three analyses of the gene product. The western immunoblot was most often discordant accounting for 6% of the 10% of analyses which were not in agreement with the other two assays of gene expression.

All of the immunohistochemical assays in this comprehensive analysis were performed on frozen tissue sections in contrast to most other studies in which immunohistochemical assays were performed on paraffin-embedded tissue sections. Since paraffin blocks from many of the cases in the comprehensive study were readily available we decided to compare the immunohistochemical assays performed in frozen with immunohistochemical assays performed in formalin-fixed, paraffin-embedded tissue sections. We found a consistent reduction in the amount of immunostaining observed in paraffin tissue sections compared to that observed in frozen tissue sections on a cases by case basis (Slamon et al, 1989b). This was most apparent in breast cancers with 2- to 5-fold amplification of HER-2/*neu* and low-end overexpression of the protein product. Many of these cases, although moderately immunostained in frozen sections, were not immunostained at all in paraffin sections. This observation indicates that the sensitivity of the immunohistochemical assay must be carefully characterized with each antibody before it is used in studies of archival paraffin-embedded tissues to assess the relative loss of sensitivity. Reliance on paraffin-embedded tissues without characterizing the loss of immunoreactivity may be responsible for some of the discrepancies present in the literature.

We were also interested in identifying other types of malignancy in which HER-2/*neu* might be amplified and overexpressed. To this end we screened DNA derived from 60 non-small cell carcinomas of the lung, 40 colon carcinomas, 35 neuroblastomas of the adrenal gland and 18 ovarian carcinomas. Amplification was observed only in 6 cases of ovarian carcinoma. Based on this observation we expanded the number of ovarian carcinomas analyzed.

AMPLIFICATION AND EXPRESSION OF HER-2/*neu* IN OVARIAN CANCER.

Amplification and expression of the HER-2/*neu* oncogene was characterized in ovarian carcinomas from 120 patients. 120 cases were analyzed by Southern hybridization, 67 by northern hybridization, 37 by western immunoblot and 72 by immunohistochemical staining. Southern hybridization showed that the HER-2/*neu* gene was amplified in 31 out of the 120 ovarian cancers (26%). Two to 5-fold amplification was observed in 19% and >5-fold amplification in 7% of the ovarian cancers. Expression of the HER-2/*neu* message was determined by northern hybridization. Quantization of the autoradiograms with soft laser densitometry showed that 44 cases (65.7%) had low levels; 16 cases (23.9%) had elevated levels and seven cases (10.4%) had either high or very high levels of HER-2/*neu* message. Expression of HER-2/*neu* protein product was assayed by western immunoblot analysis and immunohistochemistry. Soft laser densitometry of western autoradiograms showed that 27 cases (73%) had low levels, eight cases (21.6%) had elevated levels and two cases (5.4%) had high or very high levels of p185 protein product. Immunohistochemical assay for HER-2/*neu* showed detectable membrane immunostaining in 69 of the 72 analyzed cases (95.8%). Immunostaining was weak or moderate in 36 cases (50%), strong in 32 cases (44%) and very strong in 4 cases (6%).

Immunohistochemistry showed that HER-2/*neu* was present on the cell membranes of carcinoma cells. It was not identified in tumor stroma, blood vessels, or inflammatory cells. Weak cytoplasmic immunostaining for HER-2/*neu* was identified in the highly amplified (>5-fold) overexpressors but never without accompanying membrane staining. These cases also had strong membrane immunostaining. We interpreted the cytoplasmic immunostaining in these cases as nascent HER-2/*neu* protein being synthesized within the cell which is destined for transport to the cell membrane.

When the analyses were unblinded it was noted that overexpression of HER-2/*neu* was observed in all cases with amplification of the gene. In 50 of the 120 cases sufficient material was available for analysis of expression by at least two techniques, Northern blot and immunohistochemical analysis. Elevated levels of expression were also noted in 6 of these cases (12%) which had a single (diploid) copy level of the HER-2/*neu* gene. These results were very similar to those obtained in breast cancer.

Ovarian cancer, unlike breast cancer, has few prognostic indicators of clinical outcome. Therefore, a comparison of the survival data from these cases is of particular interest. Long-term followup was available for 87 of the 120 women in our study. There was a statistically significant correlation between gene amplification and survival with median survivals of 1879, 959, and 243 days for women having one copy, 2-5 copies and >5 copies, respectively, of the HER-2/*neu* gene in their ovarian carcinoma tissue (Table 2). A statistically significant association was also observed between overexpression by immunohistochemical assay and survival (Table 2). The results obtained with Northern and Western blot analyses also showed a negative correlation between increasing expression and survival but the results by Western analysis did not reach statistical significance due to the small number of cases analyzed (Table 2).

TABLE 2. Comparison of HER-2/*neu* Amplification and Expression in Ovarian Cancer with Median Survival of the Women.

Assay	Group	N	Median Survival (days)	Log Rank Test (P)	Cox Prop Hazards Model (P)
DNA	1 copy	64	1879	<0.0001	<0.0001
	2 to 5	17	959		
	>5	6	243		
RNA	1+	44	1960	0.0710	0.0244
	2+	16	919		
	3+ to 4+	7	531		
Protein	1+	27	1960	0.1866	0.0628
	2+ to 4+	10	959		
ICA	- to 1+	36	1960	0.0126	<0.0001
	2+	32	1093		
	>2+	4	417		

DNA = results of Southern blot analysis; RNA = results of Northern blot analysis; Protein = results of Western immunoblot anlaysis; ICA = results of the immunocytochemical assay; N = number of cases in each group. The Kaplan-Meier method and the Cox proportional hazards regression model were used for the statistical analyses. The median followup was 75 months for those patients still alive. (reproduced from Science 244: 707-712, 1989)

CONCLUSION.

There are a number of interesting similarities between primary malignancies of the breast and ovary. Not only are the malignancies arising in both sites predominantly adenocarcinomas but many of the adenocarcinomas express steroid hormone receptors, especially estrogen and progesterone receptors (Jensen et al, 1971; Holt et al, 1979; Press et al, 1985; Press and Greene, 1988). Epidemiologic studies suggest that some carcinomas of the breast and ovary may share common etiologic factors since women with breast cancer have twice the expected incidence of ovarian cancer and women with ovarian cancer have a three- to fourfold increased risk of developing carcinoma of the breast (Young RC et al, 1985). Similarities in the pathobiology of the HER-2/*neu* oncogene in breast and ovarian cancers are striking. The oncogene is amplified in approximately 25-30% of both breast and ovarian cancers. In both it is expressed in an amount proportional to the degree of amplification, with the exception of approximately 10% of cases which are single copy overexpressors. Finally, HER-2/*neu* appears to be an independent predictor of prognosis in both diseases. Since breast and ovarian cancer account for approximately one third of all new cases of cancer in women the potential clinical utility of HER-2/*neu* as a prognostic aid is considerable.

Although results from most large studies indicate that HER-2/*neu* has prognostic significance in node-positive breast cancers, conflicting results have been obtained for HER-2/*neu* as a prognostic factor in node-negative breast cancer. Preliminary studies using DNA amplification (Ro et al, 1989) or immunohistochemical staining of paraffin-embedded tissue sections (Wright et al, 1989) from lymph node-negative breast cancer indicate that HER-2/*neu* is associated with a shorter disease free interval and a shorter overall survival, while another study of lymph node-negative breast cancer analyzed by Western immunoblot indicates that expression of HER-2/*neu* does not have predictive value (Tandon et al, 1989). This is an ongoing area of controversy which can only be resolved by additional studies.

There is already strong supporting evidence from experimental work that alterations in the HER-2/*neu* gene may play an important role in transformation and tumorigenesis. Transgenic mice containing a mutated *neu* transgene driven by the mouse mammary tumor virus promotor uniformly developed breast cancer (Muller et al, 1988). Not only did both male and female mice develope breast cancer, but all breast epithelial cells appeared to be morphologically neoplastic, so all ducts without invasive carcinoma appeared to contain carcinoma in situ. Transfection and subsequent amplification/overexpression or overexpression alone of a normal HER-2/*neu* gene (Hudziak et al, 1987; diFiore et al, 1987) renders NIH3T3 cells more tumorigenic in nude mice.

HER-2/*neu* may have therapeutic as well as diagnostic utility. Since this oncogene is highly expressed in breast cancer cells of women with a less favorable prognosis and since HER-2/*neu* encodes a membrane protein with an extracellular domain, it is an excellent candidate as a target for immunotherapy. Experimental data using *neu*-transformed cell lines demonstrated that monoclonal antibodies to the extracellular domain were able to arrest the growth of the tumor in nude mice (Drebin et al, 1985). These observations in addition to the prognostic capability of HER-2/*neu* make this gene a potential target for further diagnostic and therapeutic strategies.

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GROWTH CONTROL IN LEUKEMIA

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INTRODUCTION

We have been analyzing the mechanisms of deregulated growth and differentiation that characterize the malignant hematopoietic cells in patients with chronic myelogenous leukemia (CML). CML has long been regarded as an interesting model for studies of cancer in general because it exhibits many of the features of solid tumors (clonal heterogeneity, genetic change, progression, etc.) but, because of its hematopoietic nature has been more accessible to study at the cellular, molecular and genetic level. For many years CML was also unique amongst human malignancies in its association with a particular alteration of the genome. In most cases of CML this can be visualized in metaphase preparations of the leukemic cells as a reciprocal translocation between chromosomes 9 and 22 (Rowley, 1973). This translocation results in the formation of a shortened version of chromosome 22, referred to as the Philadelphia (Ph^1) chromosome, in honour of the city where it was first identified more than 25 years ago (Nowell & Hungerford, 1960). The rearrangement of chromosomal material that underlies the formation of the Ph^1 chromosome brings into juxtaposition most of the c-ABL proto-oncogene from chromosome 9 and a gene now designated as the break-point cluster region gene (BCR-1) on chromosome 22 (de Klein et al., 1982; Groffen et al., 1984; Hermans et al., 1987). The creation of this new BCR-ABL "fusion" gene results, in turn, in the transcription of a new mRNA species (Hermans et al, 1987; Collins and Groudine, 1983; Gale and Canaani, 1984), which directs the synthesis of a new gene product. Interestingly, the product of the BCR-ABL gene resembles that encoded by v-ABL (the oncogene of the Abelson murine leukemia virus) in its possession of tyrosine kinase activity (Konopka et al., 1984; Clark et al., 1987; Kurzrock et al., 1987; Chan et al., 1987). The role of the BCR-ABL kinase in maintaining the malignant state is still a mystery. Indeed direct evidence for its ability to transform normal human hematopoietic cells is still lacking. However, the BCR-ABL gene does meet the criteria of an oncogene in transformation assays using murine hematopoietic target cells (McLaughlin et al., 1987), and it can be anticipated that in man the BCR-ABL gene product will eventually be shown to contribute to the phenotype of the malignant cells.

One of the hallmarks of the myeloproliferative diseases in general, and CML in particular, is the continuous production of essentially normal, albeit clonal, mature blood elements for many months, even years. In CML the median duration of the initial chronic phase which is characterized by this minimal pertubation of hematopoietic differentiation processes is approximately 3.5 years (Sokal, 1987), although inevitably there is progression of the disease to a more acute and rapidly fatal phase. Thus, the chronic phase of CML offers an opportunity to address questions about the regulation of various leukemic progenitor cell types because these can be defined using established in vitro colony assay procedures that depend on the faithful expression of normal hematopoietic proliferation and differentiation programs.

The most obvious clinical feature of CML is a marked increase in circulating granulocytes and their immediate precursors, in association with a hypercellular marrow showing very active myelopoiesis. Although the granulopoietic lineage appears to be the most affected in terms of increased output of mature cells into the circulation, erythropoiesis and megakaryocytopoiesis are also clonal, as demonstrated by both cytogenetic and G6PD isoenzyme studies (Whang et al., 1963; Dubé et al., 1984; Raskind and Fialkow, 1987). More recently it has also been shown that the malignant clone usually contributes at least some cells to the peripheral B cell population (Raskind and Fialkow, 1987; Bernheim et al., 1981). This suggests that the cell in which the Ph^1 chromosome first appears is typically a very primitive hematopoietic cell with the potential for some lymphoid as well as myeloid differentiation.

CLONOGENIC CELLS IN CML

We have sought to determine why granulopoiesis appears to be amplified more than other lineages in CML, and how the malignant clone comes to dominate the hematopoietic system and suppress normal cells. To address the first question we have measured the numbers of hematopoietic colony-forming progenitor cells at different stages of differentiation on two different lineages: the erythropoietic and the granulopoietic pathways. Unfortunately absolute values cannot be derived from assays of human marrow aspirate samples because of the contamination of each aspirate with a variable amount of peripheral blood that cannot be reliably quantitated. Thus marrow data can only provide measurements that compare one cell type to another.

Absolute values for circulating progenitor compartments can, however, be readily derived for circulating progenitor compartments since defined volumes of peripheral blood can be processed and assessed. Such measurements have revealed that all types of hematopoietic progenitors are typically increased in CML patients (Eaves and Eaves, 1979; Goldman, et al., 1980; Vainchenker et al., 1982; Lepine and Messner 1983). In the erythroid lineage, this amplification is perpetuated right into the CFU-E compartment (Eaves and Eaves, 1979), a stage of differentiation thought to immediately precede the proerythroblast. Why the large numbers of CFU-E present in CML patients fail to generate an increase in the numbers of more differentiated erythroid cells is not clear. One possibility is that they are intrinsically defective in a fashion that results in their premature failure to complete the erythroid differentiation process. However, it is also possible that certain extrinsic regulatory mechanisms (either normal or inappropriately suppressive) may be operative. Further studies will be required to evaluate these different possibilities.

Comparison of the ***ratio*** of the more primitive erythropoietic (BFU-E) and granulopoietic (CFU-GM) progenitors present in the circulation of a large number of individual CML patients has shown this to be, on average, unchanged from normal, in spite of the very significant amplification in progenitor numbers that may be seen. Essentially normal ratios are also found when marrow progenitors are assessed (Eaves and Eaves, 1979; Eaves et al., 1980). Additional studies of our own and others have demonstrated that the vast majority of these hematopoietic progenitors are Ph^1-positive and belong to the neoplastic clone (Dubé et al., 1984). Thus, there is no evidence to suggest that the multipotential leukemic stem cells in CML exhibit an abnormally increased probability of entering the granulopoietic pathway at the time their differentiation potential undergoes lineage restriction. The specific mechanisms accounting for the excessive granulocyte production that occurs in CML are therefore more likely due to regulatory defects that influence the kinetics of more differentiated, "committed", leukemic granulopoietic progenitor populations.

One mechanism by which mature cell output might be increased would be a change leading to a decreased transit time for cells to pass through successive stages of granulopoiesis. To investigate this requires the use of a method for measuring the turnover characteristics of individual types of hematopoietic progenitor cells. Standard labelling or flow cytometric procedures are not suitable because the cells of interest are rare members of the total marrow or blood cell populations and cannot yet be identified by unique morphological or surface antigen features.

A related type of measurement is that referred to as the tritiated-thymidine suicide technique (Becker et al., 1965). This yields data on the proportion of cells in any given clonogenic population that are in S-phase at the time of assessment. Thymidine suicide studies have shown that in normal adults, all types of circulating clonogenic hematopoietic progenitors are quiescent, as are the most primitive progenitors in the marrow, the latter being distinguished by their very high proliferative potential and hence ability to generate very large colonies in semi-solid media (Cashman et al., 1985). Application of the thymidine suicide technique to blood and marrow samples from CML patients has shown that all clonogenic leukemic progenitor classes are continuously cycling regardless of their type or location in vivo (Lepine and Messner, 1983;

Eaves and Eaves, 1987). Thus progenitors that are normally in G_0 appear to be deregulated in CML and one might speculate that the mechanism by which normal hematopoietic cells are rendered quiescent is a target of the transformation process.

The lack of proliferation control exhibited by primitive Ph^1-positive progenitors is consistent with the multi-lineal expansion of the Ph^1-positive clone and its ability to dominate the entire hematopoietic system. On the other hand it does not, of course, help to explain why amplification of hematopoiesis beyond the early progenitor compartments is largely confined to the granulocyte-macrophage lineage.

Abnormal responsiveness to growth regulators that stimulate granulopoiesis in vitro has been evaluated as another possible mechanism for the increased granulopoietic activity seen in CML. Interestingly, no evidence of alterations in the granulopoietic progenitor compartments in CML has been found (Metcalf, 1977). On the other hand, an abnormality in the regulation of erythroid cell growth can often be demonstrated. This is manifested as an ability of terminally differentiating erythroid cells in CML to survive and complete their differentiation in vitro in the absence of erythropoietin (Eaves and Eaves, 1979). As a result some progenitors will form colonies of hemoglobinized erythroblasts in cultures to which no erythropoietin is added, in contrast to assays of normal cells where under the same conditions such colonies are not seen. Erythropoietin-independent erythropoiesis in vitro is not, however, a constant finding in all CML patients, nor even in the same patient analyzed at different times, nor has any obvious relationship to duration of disease, treatment, total white count, etc., emerged. Erythropoietic cells that show a decreased requirement for erythropoietin have also been found in other types of myeloproliferative diseases (Eaves et al., 1980), but only in polycythemia vera has their presence been established as a consistent and hence diagnostically definitive feature (Eaves et al., in press).

LONG-TERM CULTURES

From the studies of CML progenitors identifiable in colony assays it has thus become clear that the mechanisms that hold the majority of very primitive normal cells in G_o are ineffective or can be bypassed by their Ph^1-positive counterparts. To investigate further the basis of the normal control mechanism, and to provide a starting point for understanding how it may be overcome in CML, a system in which this mechanism can be established and analyzed in vitro was required. Colony assay systems are not well suited for this, as they are designed to maximally stimulate the proliferation and differentiation of individual primitive cells rather than modulate their turnover. What would be ideal to investigate the latter would be a culture system that reproduces in vitro the supportive and local regulatory functions of the marrow microenvironment. The long-term bone marrow culture system developed by Dexter and colleagues (Dexter et al., 1977) initially for murine marrow, and later successfully modified for human marrow (Gartner and Kaplan, 1980; Coulombel et al., 1983), appears to have many of these features (Eaves et al., 1987).

To investigate the behaviour of leukemic cells in this system we began by studying a series of untreated CML patients. The initial goal was to evaluate and quantitate separately the numbers of Ph^1-positive and Ph^1-negative hematopoietic cells present after increasing periods of culture. Although Ph^1-negative hematopoietic cells are rarely detectable in CML patients because of the excessive numbers of Ph^1-positive cells, they can be consistently found in selected patients with minimally expanded Ph^1-positive clones. Such patients have WBC counts that are only slightly elevated ($<40,000$ per mm^3) in the absence of any treatment and their residual normal hematopoietic progenitors can usually be readily demonstrated (Kalousek et al., 1984). Therefore we included some patients in this latter category in the initial CML patients studied.

The results obtained from assessment of these long-term marrow cultures were unanticipated. In every case the Ph^1-positive hematopoietic cells declined, usually quite rapidly, so that within 4 to 6 weeks after placing the CML marrow in culture Ph^1-positive progenitors were no longer detectable (Eaves et al., 1985). In contrast,

Ph^1-negative hematopoietic cells co-existing in the same marrows fared better and thus frequently became the dominant population, even in cases where the number of Ph^1-negative cells in the initial marrow aspirate had been below the limit of detectability.

As yet we still do not understand the basis of this apparent differential survival of normal and Ph^1-positive cells in the long-term marrow culture system. However, subsequent studies revealed a similar phenomenon in long-term cultures initiated with marrow from patients with newly diagnosed acute myeloid leukemia (AML) (Coulombel et al., 1985). These findings are now being tested as a strategy for purging autologous marrow autografts to support the use of marrow-ablative treatment in attempts to cure both CML (Barnett et al., in press) and AML (Chang et al., 1986) patients.

Our initial "failure" to maintain Ph^1-positive hematopoiesis in long-term marrow cultures prompted further studies along two separate but now converging directions. The first was to focus in greater depth on analyzing the mechanisms regulating the proliferation and differentiation of normal hematopoietic cells in this system. The second was to explore various modifications that might make it possible to maintain Ph^1-positive cells for a sufficient period to allow their regulation to be studied also.

To pursue the first of these questions, thymidine suicide studies were undertaken to assess the cycling characteristics of the various types of progenitors in the non-adherent and adherent fractions of long-term marrow cultures established from normal individuals. These studies revealed a pattern of progenitor proliferation control in the adherent layer of established long-term cultures (i.e. >3 weeks old) that closely resembled that seen in the marrow in vivo. At the end of each week of culture, just prior to the usual medium change, the same classes of primitive progenitors that are quiescent in the marrow were found to be quiescent in the adherent layer of long-term marrow cultures (Cashman et al., 1985). However, following the addition of new medium (the active component of which we now know to be a factor present in horse serum), these cells are activated, albeit transiently, into cycle (Cashman et al., 1985). We subsequently showed that their return to a quiescent state 5-7 days later only occurs when they are in close proximity to marrow stromal cells (Eaves et al., 1986), and that this "shut-down" of their turnover appears to be mediated by TGF-β produced or concentrated in the adherent layer (Eaves et al., 1988). Re-entry of primitive hematopoietic cells into S-phase requires either their dissociation from the adherent layer, a process that occurs continuously and accompanies the further differentiation of these cells (Slovick et al., 1984), or the addition of any one of a number of cytokines with known abilities to activate mesenchymal cells. Cytokines with this property include PDGF, Interleukin-1, Interleukin-2 and TGF-α, all of which presumably achieve their effects on the hematopoietic elements by indirect mechanisms involving the mesenchymal components of the long-term culture adherent layer (Eaves and Eaves, 1988b). One of the pathways by which this indirect effect might be achieved could be through the activation of stromal cell derived hematopoietic growth factor production. Recent studies have provided some support for this hypothesis (Eaves and Eaves, 1988a; Humphries et al., 1988). Additional work with purified cell populations and better strategies to look at the role of specific growth factors should facilitate further investigation of this model.

In order to allow parallel studies of Ph^1-positive progenitors in the same type of culture system we tried a number of different approaches. One was to simply try and selectively increase the number of primitive Ph^1-positive cells added initially. Although the cells responsible for initiating long-term hemopoiesis have not yet been well characterized and, in particular, their degree of overlap with clonogenic cells is not yet clearly defined, it seems likely that these are closely related populations. The tremendous elevation of clonogenic cells seen in the peripheral blood of CML patients with very high WBC counts suggested that such samples might also contain significantly higher numbers of more primitive Ph^1-positive cells, including those responsible for maintaining the continuous production of clonogenic Ph^1-positive progenitors. CML peripheral blood was therefore substituted for marrow as a source of Ph^1-positive hematopoietic cells for culture initiation. Since the mesenchymal cells that give rise to the critical adherent stromal layer of long-term marrow cultures do not circulate (Eaves et al., 1986), an alternative source of this component of the culture system had to be used. For this we chose pre-established long-term culture adherent layers, obtained by initiating standard cultures with normal marrow. Just prior to being seeded with the CML peripheral blood cells, these normal adherent layers were subcultured and irradiated to reduce and inactivate any hematopoietic elements they would still also contain.

This "reconstruction" strategy using CML blood as a source of Ph^1-positive progenitors proved to be just the answer for our experimental needs and production of Ph^1-positive progenitors could be readily demonstrated in these cultures for many weeks (Eaves et al., 1986). Assessment of the cycling behaviour of the primitive Ph^1-positive progenitors provided further evidence of the utility of this modified culture system as an in vitro model of CML. As in vivo, all classes of Ph^1-positive progenitors were found to be rapidly proliferating regardless of the age of the culture or the time of assessment since the previous medium change (Eaves et al., 1986). Failure of the primitive leukemic progenitors in the adherent layer to be down-regulated, which occurs even in analogous reconstructed cultures of normal blood on irradiated established feeders (Eaves et al., 1986), was a particularly interesting finding. It indicated that the leukemic cells themselves were responsible for their defective behaviour, since the adherent layers used had all been derived from normal individuals. Recent studies have indicated that this defective behaviour cannot be ascribed to an inability of Ph^1-positive progenitors to be down-regulated by TGF-β since this response mechanism does not appear to be altered (Cashman et al., 1989). Whether the abnormal proliferative activity of the leukemic cells is due to an intrinsic change that affects a different signal transduction pathway or modulates the environment of the cells leading indirectly to a heightened self-stimulation awaits further investigation.

SUMMARY AND CONCLUSIONS

Quantitative assays for individual hematopoietic progenitors have provided a powerful approach to the assessment of changes in the absolute and relative numbers of primitive cells in the affected tissues of individuals with CML. These techniques have also been usefully coupled to other ways of assessing these cells to confirm their malignant nature by cytogenetic analysis and to establish and characterize abnormalities in the regulation of their numbers and turnover. A culture system that allows primitive normal and leukemic hematopoiesis to be maintained for many weeks has been developed. This system has been found to reproduce the cardinal features of primitive hematopoietic cell regulation in vivo and its use has allowed important progress to be made in characterizing the molecular basis of stromal cell regulation of primitive hematopoietic cells. Nevertheless, a large gap still remains between our knowledge of the genes that are altered in CML (or other malignancies) and the biological changes underlying the deregulation of growth that is characteristic of malignancy in general. Further exploitation of in vitro systems that allow this deregulation to be analyzed and manipulated appears to offer promise towards bridging this gap in the future.

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THE BIOLOGY OF CANCER METASTASIS

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ABSTRACT

The formation of a metastasis entails a complex sequence of events with the end result dependent on the interaction of malignant cells with host factors. Intrinsic properties of the metastatic tumor cells, including production of proteolytic enzymes, cell surface properties, adhesiveness, and the ability to grow in a distant organ environment, act in concert to influence the tumor cells' interactions with host cells in forming metastases. Life-threatening metastases are formed only by those tumor cells that have survived all steps in a process that has been shown in many experimental studies to be a highly selective event. The results of studies on the distribution of radiolabelled mouse melanoma cells injected into syngeneic mice support the concept that the fate of tumor cells released into the bloodstream is determined by sequential and selective events, and introduces a third regulatory factor. Cells endowed with metastatic properties, isolated by cloning a heterogeneous tumor or selected from a metastasis, have a higher probability of forming metastases than cells not so endowed, yet this probability is not 100%. Metastasis should thus be considered as a selective, sequential and stochastic process. Interuption of the process at any stage will prevent the formation of metastatic disease. Hence, a better understanding of the metastatic process will provide the basis for rational approaches for the prevention or destruction of this most fatal aspect of cancer.

INTRODUCTION

Metastasis is defined as the transfer of the casual agent of disease from a primary focus to distant sites, via blood or lymphatic vessels. The cellular origin of cancer metastases, rather than infection of the secondary sites by circulating humoral factors, was established in the nineteenth century. Invasion through tissues and movement around the body are however, properties that are not unique to cancer cells; the transformation event does not initiate a completely novel phenotype not otherwise seen in normal cells, or at some time in development. Properties comparable to the invasion and metastasis of malignant neoplastic cells are characteristic of some normal cells, such as lymphocytes and macrophages, and resemble the ordered movements of embryonic cells. However, whilst normal cell migration is tightly regulated by cellular interactions, cancer metastasis is a disordered movement of cells that have circumvented the homeostatic mechanisms maintaining order in the societies of cells within tissues. The growth of metastases that are resistant to therapy continues to be the primary cause of death of cancer patients (Fidler, Balch 1987).

A significant obstacle to the successful treatment of metastatic disease is the biological heterogeneity that may be present in the primary tumor and the metastases, which are in many instances established before a diagnosis of due cancer is made (Heppner, 1984; Fidler, Balch, 1987). At the time of diagnosis, the tumors contain multiple sup-populations showing a wide diversity in biochemical and biological characteristics, including cell surface molecules and receptors, expression of proteolytic enzymes, karyotpic markers, sensitivity to chemotherapeutic drugs and provisity to invade and metastasize. These last two categories define the cellular sub-populations whose survival and continued growth after therapy can lead to elapse and eventual death with disseminated cancer.

New approaches for the treatment of residual metastatic disease will be forthcoming only when we gain a better understanding of the biology of cancer metastasis, and elucidate the mechanisms by which tumor cells diversify, disseminate and grow in sites distant from the primary neoplasm.

THE PATHOGENESIS OF METASTASIS

An important theme arising from the experimental analysis of metastasis is that this is a sequence of discrete cellular events. However, the formation of metastases requires that all of the events are "successful";

failure at any point in the sequence aborts the process. (Poste and Fidler, 1979). The proliferation of cancer cells may initially be slow, and the lesions may not be manifest until many years after the carcinogenic transformation. The continued growth of tumors greater than 1-2 mm diameter requires the establishment of a blood supply, mediated by factors released from the tumors cells (Folkman and Klagsbrun 1987). Expansive growth of the vascularised tumor, and invasion of blood vessels and lymphatics result in the shedding of tumor cells or clumps of cells into the circulation. The elaboration of proteolytic enzymes such as heparanases and collagenases is associated with the metastatic ability of tumor cells (Liotta, 1986, Nakajima et al, 1987). The blood is considered an unfavorable environment for tumor cells (Weiss, 1986) and therefore survival in the circulation is the next rate limiting step in the metastatic sequence. Arterial flow is rapid, exerting shear forces on the cells in transit, which are then impacted in organ capillary beds. While mechanical factors are important in the course of blood-bone metastasis, tumor cell properties that can reduce the risk of cell death in transit have been identified. Tumor cells that can form aggregates, either homotypic or heterotypic with platelets or lymphocytes have an increased chance of surviving to form metastases (Lotan and Raz, 1983, Glaves, 1983).

Another important determinant of metastatic potential is the interaction between circulating tumor cells and capillary endothelium. This includes the non-specific mechanical lodgement of tumor and emboli cells in the narrow capillaries, and also more stable adhesions between the tumor cells and the endothelial cells. Where metastases are formed may depend at least in part on specific adhesion of tumor cells to endothelium in different organs (Nicolson, 1988). The binding of tumor cells to the endothelial cells can lead to their retraction and exposure of the underlying basement membrane, to which the metastatic cells can also bind with great affinity (Kramer et al, 1980).

The extravasation of arrested tumor cells is thought to be mediated by mechanisms similar to those responsible for the initial release of the cells into the circulation. For rodent mammary tumors the metastatic potential correlates with the ability of tumor cells to secrete basement membrane degrading enzymes (Tarin et al, 1982, Nakajima et al, 1987). The heparanase activity of mouse melanoma cells corresponds with the lung colonizing ability of the cells after intravascular injection, and blocking this enzymes's activity by pre-incubation of the cells with heparin also reduced the numbers of lung metastases (Irimura et al.1986). These, and other findings, suggest that different tumors may have different enzymatic mechanisms for achieving the same end; the invasion of basement membrane and establishment of micrometastases in organ sites distant from the primary tumor.

The significance of the multi-step nature of metastasis is that a tumor cell with metastatic potential may not be recognized by a single attribute, such as the ability to adhere to endothelial cells *in vitro*, or the production of proteolytic enzymes. Instead, the combination of properties that enable a small, selected proportion of cells released from the primary tumor to form metastases needs to be recognized, so that therapies can then be focused on one of the rate-limiting steps (Fidler, 1984).

The conclusion that metastasis is a selective process is based on numerous studies with different experimental tumor systems, using techniques that enrich tumor cell populations for metastatic potential (Fidler, 1984, Talmadge and Fidler, 1982, Raz et al., 1981). The clearest examples are those in which variants originating from metastases show enhanced metastatic capability compared with the non-selected tumor (Talmadge and Fidler, 1982). Experimental studies of different aspects of the metastatic sequence, using two murine melanomas and selected metastatic variants will now be discussed in more detail.

Distribution and fate of intravenously released melanoma cells

Labeling tumor cells by *in vitro* incubation with [^{125}I] iodo-2'-deoxyuridine ([^{125}I] IdUrd), and following the distribution patterns of the cells injected *in vivo*, by monitoring the radioactivity in different organs is an elegant technique for studies of metastasis (Fidler, 1970). Using this technique it has been realized that only a small proportion, maybe less than 0.1% of the cells injected intravenously (i.v.) into an animal actually survive and form metastases (Fidler, 1970), leading therefore to the conclusion that the metastatic process is very inefficient (Weiss, 1986).

Six variants of the murine melanoma K-1735 were studied, either clonally isolated (Clones 2,4,10,19, 23) or selected from a lung metastasis (M-2) (Fidler et al, 1981,Talmadge and Fidler, 1982). The metastatic properties of each variant had been previously evaluated (Aukerman et al. 1986). The variants were labelled by overnight incubation of log-phase cultures with 0.3 µci/ml [^{125}I]IdUrd, and the cells were injected i.v. into groups of syngeneic C3H/Hen mice. Mice were killed at intervals after the injection, organs collected and washed in 70% ethanol, to remove non-cell associated ^{125}I, released from dead tumor cells. One advantage of using [^{125}I]IdUrd as a cell label is that it is not reutilized by normal tissues (except the thyroid), and thus the radioactivity remaining in tissues is a measure of the viable input cell content (Fidler, 1970).

The length of time that cells are circulating in the blood following i.v. injection is brief. The majority (up to 80%) of the K-1735 melanoma variant cells were arrested in the lungs within 10 minutes, the first time point assayed. These findings confirm those of other studies showing that most cells arrest initially in the first capillary bed downstream of the injection site. At 10 min and 1 hr after injection there were no significant differences among the six K-1735 cell lines in the proportions of cells trapped in the lungs. By one day however, clear differences emerged in the numbers of cells surviving in the lungs, separating the cell lines into two distinct groups (Table 1). For low metastatic clones, (C-10, C-19, and C-23) the number of cells surviving in the lungs was barely detectable. However, the metastatic cells (M-2, C-2, and C-4) were still detected in the lungs, in decreasing numbers, up to 14 days after injection. The differences were not related to radiotoxicity of the [^{125}I]IdUrd to the low-metastatic cells, as all labeled variants showed similar plating efficiency and growth _in vitro_ (Price et al. 1986). Instead the ability to form experimental lung metastases was directly related to the retention and survival of the labeled cells in the lungs. However, even for the highly metastatic cells, isolated by _in vitro_ cloning (C-2, C-4) or from a metastasis

TABLE 1

Distribution and fate of K-1735 melanoma cells in the lungs of mice following intravenous injection.

Time after injection[a]	Percent of input counts remaining[b]					
	M-2	C-4	C-2	C-10	C-19	C-23
1 hr	39.7	82.5	83	45.5	68	62.6
24 hr	14.9	7.2	17	0.03	1.01	0.1
72 hr	12.0	1.7	6.6	0	0.01	0.03
168 hr	8.6	2.3	0.3	0	0.01	0.03
Experimental Metastasis[c]	300 (200->300)	250 (112->300)	100 (68-150)	0 (0-1)	0 (0-2)	0 (0-5)

Footnotes:

[a] 10^5 labeled cells injected

[b] Percent of radioactivity (mean of 5 mice) determined after 3 daily washes of lungs in 70% ethanol.

[c] Number of lung metastases, [median and range in parentheses] after i.v. injection of 10^5 cells, counted by 35 days after injection.

(M-2) only a small proportion (2-3% of the input cells were responsible for the growth of lung metastases. Events taking place soon after injection and arrest led to the elimination of practically all the low-metastatic K-1735 cells, and most of the highly-metastatic cells. This raises the question of why the majority of the metastasis-selected K-1735 M-2 cells did not survive to produce metastases. Although many studies have shown that intrinsic and heritable tumor cell properties are essential for metastasis, allowing the isolation of selected highly metastatic variants, additional factors contribute to determining the outcome of blood-bone metastasis. The propensity of circulating tumor cells to survive and form experimental metastases can be influenced by factors such as stage of cell cycle, integrity of the cell membrane, damage arising from experimental manipulations, and shear forces in the blood-stream (Fidler, 1978). These are factors that would eliminate tumor cells in an arbitrary manner, regardless of their potential to proliferate in an organ environment. The results that cells from the heterogenous K-1735 melanoma exhibit distinct and stable metastatic properties reaffirms the conclusion that the formation of metastases is a selective process. The additional data from the radiolabeling studies showing that not every cell from a metastatic population survives to make metastases, suggests that even these specialized cells are subject to chance events during the course of dissemination. The probability of tumor cells possessing certain properties, for example resistance to natural killer cell lysis (Gorelik et. al. 1979) or ability to degrade collagen (Tarin et al. 1982, Nakajima et al.) to survive and produce metastases is much greater than the probability of cells not so endowed. Yet, all cells released into the blood encounter chance events such as shear forces, and impaction in capillary beds of organs that will not support growth of metastases. These cumulative results therefore suggest that the process of metastasis is regulated by three major factors; it is sequential, selective and it may also contain stochastic elements, i.e. involving chance or probability.

The influence of the organ environment on metastasis.

Transit time of tumor cells in the blood is very short compared with the longer periods during which cells arrested in organ parenchyma proliferate to form metastases. In murine melanoma systems 2 to 3 weeks may elapse between the time of cell arrest in the lungs and the detection of metastases. In some human cancers years may pass between initial removal of the primary lesion and detection of metastases (Willis, 1973). Whether factors in the organ environment may influence the proliferation of metastatic cells is a fundamental issue in attempts to understand why tumor cells form metastases in some organ sites and not others.

The arrest of viable metastatic cells within an organ does not inevitably lead to the growth of metastases. The [^{125}]IdUrd labeling studies show that cells released intravascularly arrest in many different sites, but do not grow in all organs thus involved (Price et al. 1986). Organ sites where metastases form can be characteristic for the particular tumor. For example, a murine reticulum cell sarcoma (M5076) forms liver metastases and few lung metastases after intravenous inoculation despite the initial arrest of most of the i.v. injected cells in the lung (Hart et al., 1981). Metastases from M5076 tumors growing in muscle or the subcutis are also found only in liver, thus showing a consistent organ tropism. Studies such as these are an experimental counterpart of the analyses of autopsy cases of human cancer metastasis, and provide a verification of Paget's seed and soil hypothesis.

Distinct patterns of metastasis characterize some human tumors, and the patterns cannot be explained in all instances by rates of blood flow to the sites of metastasis, e.g. the seeding of bronchial carcinoma cells in the adrenal glands and cerebellum (Willis 1973). From a study of over 700 breast cancer patients Paget concluded that "if we can trace any sort of rule or sequence in the distribution of cancer...then the remote organs cannot be altogether passive" (Paget, 1889). It is known that local trauma or irradiation appears to enhance metastasis, thus providing more evidence that the organ environment may influence the growth of cancer metastases (Milas and Peters, 1984).

Tumor cell-host cell interactions that follow extravasation of metastatic cells into organ parenchyma will last considerably longer than the interactions taking place in the circulation. However, little is known about the influence of the organ environment on metastasis. Organ specificity of metastasis has been related to preferential adhesion of tumor cells to explants of mouse organs, and also to capillary endothelial cultures obtained from different organs (Nicolson, 1988). Another approach is to examine the effectiveness of organ-derived factors in supporting (or suppressing) the growth of metastatic cells. The studies of Horak et al. (1986) and Nicolson and Dulski (1986) showed that tumor cell growth-stimulating factors are present in media conditioned by the normal organs in which the same tumors can grow as metastases. Extrapolating these results to the intact organism suggests that factors present in different organs may support the growth of disseminated tumor cells and hence lead to patterns of organ-specific metastasis (Tarin 1985).

A highly metastatic variant of the B16 melanoma was isolated after ten cycles of i.v. injection and recovery of lung metastases. The resulting B16-F10 cells had thus survived both the blood-stream and the organ environment phase of the metastatic process, eliminating the non-metastatic cells from the original (F1) population (Fidler, 1984). To test whether growth in an organ environment alone would alter the metastatic phenotype of a low-metastatic tumor, B16-F1 cells were co-cultured with organ fragments of newborn mouse lung or kidney. The cultures were maintained in low serum conditions in which tumor cells remained viable but did not proliferate actively (Price et al. 1988). After seven days, the cells were recovered by collagenase dissociation and the surviving tumor cell fraction expanded in monolayer culture. The co-culture procedure was repeated 6 times, resulting in variants called B16-Lung6 and B16-Kidney 6. When these cells were injected into syngeneic mice it was found that their metastatic ability was the equivalent of the B16-F10 cell line, selected by a combination of *in vivo* and *in vitro* steps (Table 2).

TABLE 2
Metastatic behavior of organ-selected B16 melanoma variants.

	Organ Site[b]			
Cell line	Lung[a]	Kidney[b]	Ovary[b]	Adrenal[b]
B16-F1	14(0-51)	1	2	4
B16-F10	65(7-114)	1	14	1
B16-Lung 6	109(27-144)	0	11	0
B16-Kidney 6	62(20-159)	0	12	3

5 X 10^4 cells were injected into the lateral tail vein of C57BL/6 mice. Metastases were counted 21 days later.

[a] Results for experimental metastases are shown as median (and range) from 2 experiments of 10 mice per group per experiment.

[b] Number of mice with experimental metastases in this organ out of 20 injected.

Metastases formed primarily in the lungs from subcutaneous tumors and also direct i.v. injection (Price et al. 1988), but the incidence of ovary metastases was also increased in mice injected with the three "selected" cell lines. This, and the observation that the B16-Kidney 6 cells were not metastatic to the kidney, show that tumor cells with specific organ trophisms were not selected by this study. However, the results do show that repeated confrontation of tumor cell and organ environment can lead to the isolation of tumor cell populations with enhanced metastatic properties (Price et al., 1988). These factors, in combination with specific interactions of tumor cells with capillary endothelial cells may determine the organ-location of metastases (Nicolson, 1988). One important consequence of a metastasis selective influence of the organ in which the lesion is growing is that the metastases themselves may continue to disseminate tumor cells, to more locations or different organs, possibly at a greater rate than occurred from the primary tumor (Weiss, 1986).

The role of activated ras oncogenes in metastasis

The DNA of about 20% of specimens studied contain oncogenic sequences that can transform immortalised fibroblasts into neoplastic cells. The *ras* gene family has been extensively studied recently, as it is implicated in the oncogenic transformation of normal cells (Barbacid, 1987) and also the acquisition of the metastatic phenotype (Thorgeirsson et al., 1985, Bondy et al., 1985, Muschel et al., 1985). Introducing activated Ha-*ras* genes into tumorigenic but non-metastatic cells can induce metastatic properties, (Vousden et al., 1987, Collard et al. 1987) and expression of the p21 *ras* gene product is associated with increased production of collagenase type IV, (Garbisa et al.) 1987 increased response to motility factors (Partin et al, 1988), and alterations in cell surface features such as the structure of aspargine linked oligosaccharide. (Dennis et al. 1987)

However, further studies have shown that the incorporation of activated *ras* genes into the cellular genome does not inevitably induce the metastatic phenotype. Recent work in this laboratory, using clonally isolated non-or-low-metastatic K-1735 melanoma variants showed that while the mutant Ha-*ras* oncogene altered some aspects of the tumorigenic behavior, metastasis from subcutaneous tumors was not altered. The *ras*-transfected K-1735 cell lines showed greatly accelerated local growth in the subcutis, and also in the brain following intra-carotid artery injection. Also, when injected i.v. the cells formed experimental metastases which were more numerous and larger than those of the control cell lines. (Price et al., 1989). However, an equivalent increase in spontaneous metastasis from the s.c. tumors was not seen, leading to the conclusion that expression of the activated *ras* gene in the K-1735 melanoma cells gave a growth advantage to the cells, but did not complement the deficiencies that prevented the low metastatic K-1735 cells from forming spontaneous metastases (Aukerman et al., 1986). Since the K-1735 melanoma-*ras* transfectants did show enhanced growth proficiency in local sites and the lung (after i.v. delivery), it may be concluded that the cells were unable to escape from the primary tumor, and thus failed to seed spontaneous metastases. (Price et al. 1989).

THE CELLULAR ORIGIN OF METASTASES

In general, metastases are not seeded by cells that survive at random; instead they arise from the proliferation of specialized sub-populations of metastatic cells (Fidler, 1984). Tumor cell variants with markedly different metastatic properties can be isolated from one cell line (an example being the K-1735 melanoma, Table 1). The first experimental proof of metastatic heterogeneity in a tumor was provided by Fidler and Kripke (1977), using the B16 mouse melanoma. Clonally isolated cell lines varied widely in their metastatic ability when injected intravenously into mice. A control subcloning procedure demonstrated that the diversity was not a sequence of the technique itself. (Fidler and Kripke, 1977). Many subsequent studies have confirmed that tumors can be heterogeneous in terms of the metastatic ability (Fidler, 1984).

Many metastases can originate from the proliferation of single cells, identified by specific chromosome markers (Fidler and Talmadge, 1986), or unique genomic insertions of plasmid vectors (Talmadge and Zbar, 1987, Kerbel et al. 1987). Different studies concur that the majority of metastases have a mono-clonal origin, and also that different metastases within an organ, or in different organ sites, can arise from distinct progenitor cells.

THE GENERATION OF HETEROGENEITY WITHIN METASTASES

Even within individual metastases of known clonal origin, diversity for a range of biological parameters can rapidly develop, creating significant intralesional heterogeneity (Poste et al., 1982), recapitulating events that had occurred in the primary lesion (Heppner, 1984). A major problem in treating established metastases can be the intra-lesional diversity resulting from the continuing evolution of new variants. One explanation for the rapid appearance of diversity within metastases is the absence of the relative stability imparted by tumor cell interactions in the primary lesion (Poste et al., 1981). These interactions are thought to limit further phenotypic diversification of the neoplastic cells. Theoretically in the absence of the polyclonal stablizing interactions, tumor cell diversification within the originally monoclonal metastasis would initially be rapid. Also, if a particular therapy kills many of the cells in a heterogeneous tumor cell population, either in a primary or metastatic tumor, this might stimulate the diversification of the remaining resistant sub-populations; thus an iatrogenic stimulation of heterogeneity (Fidler and Balch, 1987).

IMPLICATIONS OF CANCER CELL HETEROGENEITY FOR CLINICAL TREATMENT

The rapid generation of tumor cells with altered phenotypes can result in the emergence of variants that are resistant or have reduced sensitivity to a particular agent (Nowell, 1976, Nicolson and Lotan, 1986). The potential for iatrogenic stimulation of diversity within the metastatic cells surviving a particular treatment makes it unlikely that single or multiple treatment regimens will eliminate all neoplastic cells. The use of repeated cycles of cytotoxic therapy for example, has been seen to result in the emergence of resistant populations. Suggested improvements of such schemes include reducing the period between successive treatment cycles, when the resistant cells would be growing and to use additional modalities to inhibit the tumor cell diversification, possibly using promotors of differentiation, growth factors, or hormones (Nicolson and Lotan, 1986).

All therapeutic protocols, chemo-, radio-or immunotherapies, are limited by the specificity of the treatment, and whether or not 100% of the metastatic cells will be eradicated (Fidler and Balch, 1987), possibly a function of the heterogeneous response of the tumor cells. An alternative therapeutic approach is the enhancement or stimulation of host-immune mediated tumoricidal mechanisms (Fidler, 1985, Rosenberg, 1985), including lymphokine activated killer (LAK) cells and macrophages, to eliminate tumor cells that are resistant to other more conventional forms of therapy. Experimental data suggests that appropriately primed host cells can kill tumor cells, regardless of the antigen expression, or drug resistance, (Fidler 1985). Repeated confrontation of tumor cells with activated macrophages did not result in macrophage-resistant variants (Fogler and Fidler, (1985), suggesting that lysis of tumor cells by these host defense cells is non-selective and has the potential to circumvent the problems arising out of cancer cell heterogeneity and the emergence of treatment resistant variants.

CONCLUSIONS

Some important principles have emerged from experimental studies of metastasis. First, that neoplasms are not uniform; they consist of cells with heterogeneous metastatic capabilities. Secondly, the process of metastasis is not random, and last that the formation of metastases depends on properties of the tumor cells and normal host factors. The balance of the tumor cell-host cell interactions could well vary in tumors arising in different organs, or in tumors of similar origin in different patients.

Though presenting a formidable challenge, the viewpoint that cancer metastasis is not random is optimistic. A process that is sequential, selective, and stochastic is more easily understood than one that is totally random. The events regulating the complex interactions in the sequence of metastasis can be studied and eventually manipulated to the benefit of the patients.

Diversification within metastatic lesions, and tumor surviving therapy can develop rapidly, possibly as a result of the instability associated with clonal populations. In addition, metastatic cells can also exhibit high rates of spontaneous mutation. Together these result in the rapid evolution of new variants of a tumor, an alarming prospect when the currently available antineoplastic therapies fail to eradicate all cells in the metastasis or primary lesion. Protocols for developing new therapies for cancer should be based on the biologic characteristics of metastatic tumor cells.

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MACROPHAGES, METASTASIS AND IMMUNITY

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INTRODUCTION

The macrophage plays a pivotal role in the response of the host to infectious organisms. Its ability to act as a primary defence cell against pathogenic bacteria such as *Leishmania donovani* or *Listeria monocytogenes* has in part led to the suggestion that it may play a role in recognizing and eradicating tumor cells. The evidence for such a role is, however, largely circumstantial. Unlike a large number of other immune effector cells, reported defects in macrophage function have not been correlated with a susceptibility to enhanced tumor growth or increased incidence of metastasis. External manipulation of macrophage function, either by the administration of stimulators or inhibitors, has however been shown to affect tumor induction (Norbury and Kripke, 1979). Whether this modulation reflects a direct effect on the macrophage or an (unknown) consequence of activity against other effector cells is still obscure.

Unstimulated monocytes or tissue-resident macrophages do not normally kill tumor cell targets *in vitro*. The ability of normal murine and human macrophages to kill tumor cell targets after stimulation *in vitro* with Biological Response Modifiers (BRM's) such as lymphokines or muramyl peptides is firmly established (David, 1975; Taniyama and Holden, 1979; Mantovani *et al*, 1980; Fidler, 1985). The activation of macrophages *in vitro* followed by their intravenous injection results in therapeutic activity against tumor growth *in vivo*

(Fidler, 1974; Liotta *et al*, 1977). It is these observation above all others which indicate an inducible, rather than an innate, role for macrophages in the control of metastatic tumor growth.

One of the fundamental problems confronting the induction of macrophage tumoricidal activity *in situ* is the rapid elimination and/or toxicity of the majority of BRM's. This has been addressed in recent years by the application of liposome delivery systems to passively target incorporated materials to cells of the reticuloendothelial system. The group of compounds most extensively studied are the muramyl peptides, derivatives of *Mycobacteria* cell wall peptidoglycan possessing potent immunostimulatory activity (Adam and Lederer, 1984). Pharmacokinetic studies have demonstrated that muramyl dipeptides are rapidly excreted after parenteral administration (Parant *et al*, 1979). N-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP) is able to induce macrophage tumoricidal activity *in vitro*: it is singularly ineffective in inducing such activity *in situ*, almost certainly because of the prolonged incubation periods required (Sone and Fidler, 1981). Liposomal MDP is considerably more effective than free MDP in stimulating macrophage tumoricidal activity *in vitro* (Sone and Fidler, 1981), and possesses anti-tumor activity *in vivo* (Fidler *et al*, 1981). The use of derivitized lipophilic drugs which interact with the phospholipid bilayer (Page-Thomas & Phillips, 1979) has recently been applied to muramyl di- or tripeptides as a means of enhancing liposomal incorporation and activating macrophage tumoricidal activity (Phillips and Chedid, 1988a; Fidler *et al*, 1988).

Induction of macrophage tumoricidal activity *in vitro* and *in vivo* by liposomal muramyl dipeptides.

We have for a number of years studied the effectiveness of liposomes to augment the immunostimulatory activity of muramyl dipeptides *in vitro* and *in situ*. We have specifically focused on macrophages from the lungs and the liver, as these two organs account for a substantial proportion of all sites of metastatic tumor growth. Our initial studies demonstrated that the incorporation of a lipophilic MDP derivative, MDPglycerol dipalmitate (MDP-GDP) within liposomal carriers resulted in a formulation which was considerably more potent than free or liposomal MDP in inducing murine alveolar macrophage tumoricidal activity against B16 melanoma target cells *in vitro* (Table 1), and which possessed antitumor activity against pulmonary tumors of the same tumor cell line (Phillips *et al*, 1985).

TABLE 1. Relative potency of free and liposomal MDP in inducing alveolar macrophage tumoricidal activity *in vitro*.

MDP preparation	Relative potency
MDP	1.0
Liposomal MDP	1.73×10^3
Liposomal MDP-GDP	2.4×10^5
Freeze-dried Liposomal MDP-GDP	1.8×10^6

(Data taken from Phillips *et al*, 1985)

The minimum effective dose (MED) for the induction of macrophage tumoricidal activity *in vitro* or *in situ* for a number of muramyl dipeptides clearly demonstrated the enhanced efficacy of liposomal incorporation (Table 2).

TABLE 2. Minimum effective doses (MED's) for the induction of macrophage tumoricidal activity.

In vitro (gm/ml)	MDP	MDPGBe	MDP(D-D)
Muramyl dipeptide	2×10^{-8}	3×10^{-7}	>0.2
Liposomal muramyl dipeptide-GDP	1×10^{-11}	1×10^{-9}	5×10^{-12}
In situ (μg/mouse)	MDP	MDPGBe	MDP(D-D)
Muramyl dipeptide	27	40	>1000
Muramyl dipeptide-GDP	3	6	15
Liposomal muramyl dipeptide-GDP	0.1	0.3	0.05

(Data taken from Phillips *et al*, 1987a)

One of the most interesting observations to come out of this study was the transformation of an inactive muramyl dipeptide - MDP(D-D) (N-acetylmuramyl-D-alanyl-D-isoglutamine) - into the most active molecule studied to date after its incorporation as the GDP-derivative within liposomal carriers.

Inhibition of pulmonary tumor growth by liposomal muramyl dipeptides.

The ability of free and liposomal muramyl dipeptides to influence the growth of a murine melanoma cell line - B16-BL6 - in the lungs of mice has been determined using a therapeutic treatment regimen (Phillips *et al*, 1985,1987a). The results of these studies unequivocally demonstrate that liposomal formulations are significantly more effective than unencapsulated hydrophilic or lipophilic muramyl dipeptides in inhibiting the growth of experimentally-induced pulmonary metastases (Table 3, Table 4).

TABLE 3. Effect of muramyl dipeptides on experimental B16-BL6 melanoma tumors in C57BL/6 mice.

Treatment, µg/mouse		Median number of lung tumors (range)
Control		50 (24-80)
MDP,	100	49 (22-68)
MDP-GDP	100	40 (15-68)
MDP-GDP	10	58 (12-74)
MDPGBe	100	56 (12-67)
MDPGBe-GDP	100	42 (12-106)
MDPGBe-GDP	10	62 (13-96)
MDP(D-D)	100	45 (11-74)
MDP(D-D)-GDP	100	29 (5-58)[a]
MDP(D-D)-GDP	10	58 (18-89)

B16-BL6 melanoma cells were injected i.v. on day 0 into groups of 10 C57BL/6 mice. Treatment was carried out on days 3,5,7,10 and 13, and the number of pulmonary tumors determined on day 21. [a]Significantly different from control-treated mice (Mann-Whitney U test,. $p<0.05$). Data taken from Phillips *et al*, 1987a.

We have routinely obtained a reduction in pulmonary tumor burden of 75-85% using liposomal muramyl dipeptides, but only when treatment is started at the micrometastatic stage. Treatment after macroscopic tumors are apparent is without effect upon tumor growth. The ability of liposomal muramyl dipeptides to inhibit tumor growth correlates with the induction of tumoricidal activity in alveolar macrophages after *in situ* treatment (IE, hydrophilic and lipophilic muramyl dipeptides are without activity whereas the liposomal formulations are active). The potency order for *in situ* activation parallels the activation of macrophages *in vitro* as well as the anti-tumor activity (Phillips *et al*, 1985, 1987a, 1988a).

TABLE 4. Effect of liposomal muramyl dipeptides on experimental pulmonary B16-BL6 melanoma tumors in C57BL/6 mice.

Treatment	μg/mouse	Median number of lung tumors (range)
Control		70 (40-98)
Control liposomes		85 (45-108)
Liposomal MDP-GDP	10	33 (45-108)[a]
Liposomal MDPGBe-GDP	10	45 (18-70)[a]
Liposomal MDP(D-D)-GDP	10	20 (12-33)[a]

B16-BL6 melanoma cells were injected i.v. on day 0 into groups of 10 C57BL/6 mice. Treatment was carried out on days 3,5,7,10 and 13, and the number of pulmonary tumors determined on day 21. [a]Significantly different from control-treated mice (Mann-Whitney U test,. $p<0.05$). Data taken from Phillips *et al*, 1987a.

Induction of Kupffer cell tumoricidal activity and inhibition of liver tumor growth by liposomal muramyl dipeptides.

The majority of i.v. administered liposomes localize in the liver, specifically within the Kupffer cells. Liposomes containing C-reactive protein or crude lymphokine preparations have been shown to inhibit metastatic tumor growth (Thrombre & Deodhar, 1984), and liposomes containing γ-interferon and MDP have been shown to be more effective than free MDP in inducing rat liver Kupffer cell tumoricidal activity *in vitro* (Stukart *et al*, 1987). Using two experimental models of liver metastasis in mice, we have investigated the potency of free and liposomal muramyl dipeptides in inducing Kupffer cell tumoricidal activity after *in vitro* or *in situ* treatment, and have determined their activity in inhibiting hepatic metastatic tumor growth.

Treatment of isolated murine Kupffer cells *in vitro* with free or liposomal muramyl dipeptides showed that liposomal MDP-GDP was significantly more potent than liposomal MDP or free MDP (Table 5). Similarly, liposomal MDP-GDP was significantly more potent than liposomal MDP in inducing Kupffer cell tumoricidal activity after *in situ* activation (Table 5).

TABLE 5. Relative potency of muramyl dipeptide in inducing *In vitro* and *in situ* activation of Kupffer cell tumoricidal activity

MDP preparation	Relative potency	
	In vitro	*In situ*
MDP	1.0	Inactive
Liposomal MDP	15.8	1.0
Liposomal MDP-GDP	2400.0	8.0

(Data taken from Phillips *et al*, 1988b)

Using a liver-specific variant of the Lewis lung carcinoma, tumor cell line H-59 (Brodt, 1986), we have shown that liposomal MDP-GDP treatment results in inhibition of liver tumor growth when either prophylactic or therapeutic regimens are used (Brodt *et al*, 1989), prophylactic treatment being the most effective. By means of surgical manipulation, it has been possible to localize B16-F1 melanoma tumor cells in the liver. Using this procedure we have determined the efficacity of free and liposomal muramyl dipeptide in inhibiting tumor growth. The results (Table 6) show that liposomal MDP-GDP treatment results in a 75-85% reduction in liver tumor burden when either prophylactic or therapeutic regimens are used. We have been unable to observe any significant effect on tumor burden with free MDP in the dose range

10-1000 µg (data not shown). In contrast, we have observed significant anti-tumor activity with liposomal MDP-GDP at doses of 0.01 µg/mouse and above. The reductions in tumor burden that we have observed corresponds favorably to that observed with IL-2/LAK therapy in the same model of hepatic metastasis (Lafreniere & Rosenberg, 1985).

TABLE 6. Treatment of experimental hepatic B16-F1 metastases in C57BL/6 mice with liposomes containing MDP-GDP

Treatment	Median number of tumors (range)
A. Prophylactic.	
Control	60 (25-159)
MDP, 100 µg	46 (18-117)
Control liposomes	78 (5-140)
Liposomal MDP-GDP, 10 µg	6 (2-11)[a]
B. Therapeutic.	
Control	230 (156->250)
MDP, 100 µg	185 (54->250)
Control liposomes	181 (101->250)
Liposomal MDP-GDP, 10 µg	62 (33-127)[a]

Groups of 5-8 mice were treated one day before tumor cell injection (prophylaxis) or on days 3,7,10 and 14 following intrasplenic tumor cell injection. The number of liver tumors was determined on day 21. [a]Significantly different from control ($p<0.006$). Data taken from Phillips *et al*, 1989a.

The results of our studies demonstrate the effectiveness of liposomes as drug-delivery vehicles for lipophilic muramyl dipeptides. The observation that enhanced macrophage tumoricidal activity in the target organ correlates with the observed anti-tumor activity indicates a more than causal relationship. The impact of other mechanisms such as the enhanced production of IL-1 from macrophages following liposomal muramyl dipeptide treatment (Phillips, 1988c) or the enhanced levels of GM-CSF following injection of liposomes containing lipophilic muramyl dipeptides (Galelli *et al*, 1989) argues for a number of effects at different levels of host defense activity. The involvement of other cell types following the stimulation of macrophages by liposomal muramyl dipeptides remains to be determined.

Liposomal vaccines for melanoma

The ability of liposomes to act as immunoadjuvants is well established: their use as potential vaccines for immunizing against tumor cell antigens is however a recent development. Liposomes are able to act as vaccines for immunization against metastatic adenocarcinoma in the rat (Steele *et al*, 1984). We have recently exploited the ability of liposomes to target compounds to macrophages in an immunological context.

Our initial studies demonstrated that liposomal incorporation of a human tumor-associated antigen - carcinoembryonic antigen (CEA) - resulted in enhanced immunological recognition in mice at doses where free CEA was ineffective (Phillips *et al*, 1987b). Using tumor-associated antigens (TAA) derived from B16 melanoma tumor cells, we have established the potency order for the induction of protective responses using free and liposomal antigen preparations. Our results show (Table 7) that immunization with liposomal TAA, especially with concomitant MDP-GDP, is singularly effective in protecting mice against a lethal tumor cell challenge (Phillips *et al*, 1988d).

TABLE 7. Effectiveness of liposome TAA vaccine in protecting against B16-BL6 melanoma.

Immunization	% Survivors	Median survival (weeks)
Control	0	3.5
Irradiated tumor cells	0	3.5
TAA 40 µg	0	3.5
Liposomal TAA 0.1 µg	30	4.8[a]
Liposomal TAA 0.1 µg plus 10 µg MDP-GDP	65	>12 weeks[a]

Groups of 10 C57BL/6 mice were immunized s.c. on days 0 and 14 with 10^6 irradiated tumor cells or with the TAA preparations. They were challenged with 10^5 tumor cells s.c. on day 28, and the length of survival determined. [a]Significantly different from control (chi-square analysis with Yate's correction, $p<0.05$). Data from Phillips *et al*, 1988d.

The potential for a sustained and directed delivery of liposome-TAA to sites of antigen processing and presentation (IE, Langerhan's cells in the skin and dendritic cells in lymph nodes) is particularly attractive and relatively unexplored as a treatment for cancer.

The results of an active-specific immunotherapy phase I study in patients with metastatic malignant melanoma using autologous tumor-associated antigens (TAA) incorporated within liposomal carriers indicate that autologous liposomal TAA preparations can be safely administered (Phillips *et al*, 1989b). Thirteen patients have been entered on the study to date and given subcutaneous (sc) injections of liposome-TAA preparations at 2-4 week intervals (Table 8).

TABLE 8 Patient characteristics and summary of therapy.

No.	Age/sex	Metastatic distribution[a]	Injections	Period (weeks)	Response
1.	42 M	Sc,Li,Lu,Sb	3	11	Mixed
2.	66 M	Sc,Li,Lu,B[c]	3	36	None
3.	52 F[b]	Sc	20	110	Complete
4.	22 M	Sc,Lu	4	14	None
5.	36 F	Sc,Sk Lu,B[c]	7	27	Mixed
6.	61 M	Sc,Li,Luc	6	32	None
7.	66 F[b]	Sc	15	102	Complete
8.	46 M	Sc,Li,Lu	3	12	None
9.	34 F	Sc,Sk,Rp	4	9	None
10.	42 F	Sc,Lu[c]	4	15	None
11.	46 M	Sc	8	26	Complete
12.	67 M[b]	Sc,Li	4	8	None[d]
13.	41 M[b]	Sc	4	8	None[d]

[a]B, bone; Li, liver; Lu, lung, Rp, retroperitoneum; Sb, small bowel; Sc, subcutaneous; Sk, skin. [b]Alive. [c]Brain metastasis developed during treatment. [d]New entry

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Clinical and laboratory monitoring did not reveal any short or long-term systemic or local toxicity. Three patients have had a complete response, and two patients had a partial response (50% or greater size reduction in one or more tumor sites) The remaining 8 patients showed no response with disease progression. Two of these 8 patients are still undergoing treatment. Significant enhancement of the ability of the autologous TAA preparations to stimulate PBL proliferation *in vitro* occurred in those patients showing a reduction in tumor mass. No proliferative responses to autologous TAA's were seen in those patients who did not respond to the liposomal TAA therapy (Table 9). The PBL mitogenic response to ConA was maintained in those patients responding to treatment, whereas the mitogenic response to ConA diminished in non-responder patients. NK cell activity did not correlate with PBL proliferation or clinical response status, whereas PBL cytostatic activity against heterologous melanoma tumor cells correlated with clinical responsiveness.

The reality of obtaining sufficient autologous tumor-associated antigenic material for the evaluation of immunization procedures is perhaps the major challenge facing immunotherapeutic intervention. In addition, such intervention in those cancers where there is no well defined or readily available tumor antigen will require the formulation and exploitation of procedures designed to maximize any impact on the immune system. Paradoxically, immunization of patients with stage II melanoma (where there is minimal tumor bulk) with TAA's will have to use small quantities of antigen: liposomes would appear to be effective in eliciting immune responses when very small quantities of antigen are incorporated.

Our data obtained from the phase I clinical trial indicates that the sc administration of liposomes containing autologous tumor-antigen preparations does not result in any acute or chronic toxicity. The clinical and immunological responses (PBL proliferation to TAA's, tumor stasis activity) observed in 5/11 patients indicates that liposomal tumor antigen therapy may be of use in an immunoadjuvant setting. These responses are encouraging, and may be indicative of a stimulated anti-tumor immunity in those patients showing clinical responses.This form of immunotherapy therefore appears to be safe and feasible candidate for a much larger phase I/II study.

TABLE 9. Peripheral blood lymphocyte responses against autologous tumor antigens and Concanavalin A.

Patient	Stimulation index[a]				
	Pre-immunization		Post-immunization		
	TAA	ConA	TAA	ConA	
1	1.0	44	15.1	38	CR[b]
2	1.0	33	1.0	6	
3	1.0	23	16.8	46	CR[c]
4	1.5	78	1.6	12	
5	1.0	67	11.8	52	CR
6	1.0	33	1.9	3	
7	1.0	46	25.0	78	CR[c]
8	1.0	92	1.0	15	
9	1.0	61	1.5	34	
10	1.0	75	1.0	17	
11	1.0	41	11.0	67	CR[c]
12	1.0	22	ND[c,d]		
13	1.0	39	ND[c,d]		

[a] Stimulation index is the proliferative response in the presence of TAA's divided by the control proliferative response. [b] CR, clinical response. [c] Alive. [d] ND, not determined.

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DEVELOPMENT OF MYELORESTORATIVE AND IMMUNOTHERAPEUTIC PROPERTIES OF CYTOKINES

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Cytokines are emerging as an important new class of agents for the treatment of cancer. However, to facilitate their development in Phase I trials and to improve their efficacy in Phase II/III clinical trials, additional information is needed on their mechanism of action (Talmadge et al, 1986). Cytokines modify the relationship between the tumor and host by regulating the host's response to the tumor with resultant therapeutic effects. We discuss herein a strategy for the preclinical development of cytokines and emphasize those agents that: 1) increase the host antitumor responses through augmentation and restoration of effector mechanisms; 2) increase the host defenses by the administration of cytokines (or recombinant derivatives thereof) as effectors or mediators of the antitumor response; or 3) increase the ability of the host to tolerate damage by cytotoxic modalities of cancer treatment. Prominant among those agents which have shown reproducible and significant clinical efficacy to date are interferon-α (hairy cell leukemia), interleukin-2 (melanoma and renal carcinoma), and colony-stimulating factors (any patient who is myelosuppressed).

It is inarguable that proteins obtained by genetic engineering may not have the complete profile of antitumor activity exhibited by the natural product or from a cytokine supernate, conversely, they may have more specific and reproducible effect(s). This was observed with the interferon-alphas where heterogeneity among natural products within the same type have been described (Gutterman, 1988). Thus it may be useful to maximally purify natural products to compare with the recombinant bioengineered analogs. In this way specific differences may be recognized, measured, and appropriate analogs developed, and clinical protocols designed if warranted using mixtures of the bioengineered molecules.

To date, immunotherapy using cytokines has had relatively limited clinical application. Nonetheless, significant therapeutic efficacy has been observed with specific and limited indications. The initial clinical studies with IFN-α, which was the first cytokine examined in the clinic, were the subject of much enthusiasm when non-randomized studies observed therapeutic activity against malignant melanoma, osteosarcoma, and various lymphomas. However, subsequent trials with IFN-α (Gutterman, 1988; Spiegel, 1987) have demonstrated significant therapeutic activity only against hairy cell leukemia, a few other types of lymphoma, perhaps renal cell carcinoma (Spiegel, 1987; Golomb et al, 1988) and most recently with AIDS and Kaposi's sarcoma (Lane et al, 1988; DeWit et al, 1988). IFN-α was first described by Isaacs and Lindemann in 1957 and the initial clinical trials with the partially purified natural product were initiated in 1978. However, the development of recombinant IFN-α (1981) was needed to provide sufficient material for studies in the number of patients required to identify the responding tumor histiotypes. It is now agreed that virtually all patients with hairy cell leukemia respond to relatively low doses of IFN-α. Thus, it has taken almost three decades to translate the concept of IFN-α as an anti-viral agent to the demonstration of routine clinical utility for neoplasia. Despite these clinical studies, the development of IFN-α is still in its early stages, and such basic parameters as optimal dose and therapeutic schedule remain to be determined (Edwards et al, 1981; Maluish et al, 1983). The mechanism of activity remains controversial since IFN-α has been shown to have dose-dependent antitumor activities in vitro, yet it is active at relatively low doses for hairy cell leukemia. The potential for immunomodulation as the mechanism of therapeutic activity with IFN-α is best exemplified by hairy cell leukemia where the full 90-95% response rate is not achieved until the patients have been on protocol for a year (Golomb et al, 1988).

Due in part to experience gained with IFN-α and γ, IL-2 and TNF there has been a rapid development, with the colony stimulating factor(s) (CSFs). The CSFs have shown marked efficacy in ameliorating the myelosuppression found in a wide variety of clinical conditions, including bone marrow transplantation, aggressive chemotherapy, and various forms of congenital or iatrogenic cytopenia. Thus, this class of cytokines, i.e., myelorestorative agents (Antman et al, 1988; Hoelzer et al, 1988; Komiyama et al, 1988; Groopman et al, 1987; Brandt et al, 1988), may prove to be the first which are broadly applicable with therapeutic efficiency.

It is readily apparent, based on the number of recombinant cytokines currently available and the rapidity with which additional ones are identified, purified, sequenced, and cloned, that the need is to understand the therapeutic mechanism of the ones we currently have and how best to translate their therapeutic potential into clinical efficacy. Concomitant with this objective is a parallel strategy to identify the pharmacological deficiencies of these agents and develop second generation agents or mimetics to address these deficiencies.

The Concept of a Hypothesis-driven Clinical Trial

In the development of chemotherapeutic drugs, clinical trials have been developed predicated on a direct relationship between increasing dose and antitumor effect(s) (Hryniuk and Bush, 1984; Tannock et al, 1988). In contrast, the effective development of recombinant cytokines, in the adjuvant setting may be independent of a direct dose response relationship, or of significant therapeutic efficacy in patients with bulky malignant disease. Under these circumstances, testable clinical hypotheses based on parameters of immune modulation, as opposed to approaches based on dosage-dependent, antiproliferative properties are needed (Talmadge, 1986; Mihich, 1986).

The development of successful immunotherapeutic protocols predicated on clinical hypotheses requires new strategies and the identification of clinically predictive tumor models and protocols (Jaffe and Herberman, 1988; Herberman, 1985). Beyond the clinical determintion of a maximum tolerated dose (MTD) as mandated by FDA requirements and chemotherapeutic traditions, it is important to determine the optimal immunomodulatory dose

(OID) in initial clinical trials. The OID is the minimal dose of a cytokine which results in the significant augmentation of effector cell activity associated with the therapeutic response. This is a dose of the cytokine which can be repeatedly administered to maintain a constant level, or repeated increases in an effector cell activity associated with therapeutic activity. This dose may be lower than the MTD, which is defined here as the largest dose of a cytokine that can be tolerated either by the clinician or the patient during clinical trials, and as such, may vary not only from patient-to-patient but also among clinical centers depending upon the physician's perception of acceptable toxicity.

The optimal therapeutic dose and protocol (OTP), yet to be determined for any cytokine in the clinic, likely depends not only on the augmentation of effector cell activity but also on the number, function, and tumor infiltration of effector cells. Furthermore, the determination of an OTP requires information on therapeutic schedule and duration of treatment. Studies of scheduling parameters have revealed that many BRMs induce host toxicity when delivered frequently at high doses. However, if the BRM is administered at either a lower level, less frequently, or by a strategy to address pharmacokinetic deficiencies, there may be a decrease in toxicity and a concomitant increase in therapeutic activity. Thus, it has not been unusual to observe a bell-shaped dose-response curve for immunomodulation in preclinical and clinical trials and for therapeutic activity in preclinical studies. This bell-shaped curve may be due to decreased immunomodulatory potential, or toxicity to the host at the higher doses.

Perhaps the best example of a hypothesis which has been evaluated in the adjuvant clinic setting, is that human recombinant interferon-gamma (rHu-IFN-y) will have therapeutic activity at an OID which is a dose lower than the MTD (Jaffe and Herberman, 1988). Preclinical studies have revealed that murine rIFN-y (rM-IFN-y) has significant therapeutic activity in models of experimental and spontaneous metastasis which occurs with a reproducible optimal dose and treatment schedule and has a bell-shaped dose response curve (Talmadge et al, 1987). Further, studies of macrophage activation in normal animals revealed that the same bell-shaped dose response curve occurred for the augmentation of this effector cell. In subsequent studies, in which tumor bearing animals were treated with rm-IFN-γ, a significant correlation was observed between macrophage augmentation in the lungs of pulmonary metastasis bearing mice and therapeutic efficacy (Black et al, 1988). This suggests that immunologic augmentation may provide an indirect mechanism for the therapeutic effect and supports the hypothesis that treatment with the MTD of r-IFN-γ may not be optimal in an adjuvant setting. The bell-shaped dose response curve for rHu-IFN-γ has been confirmed in a number of clinical studies which have documented the immunoregulatory effects (macrophage augmentation) of rHu-IFN-γ and defined an OID (Maluish et al, 1988; Kleinerman et al, 1986; Thompson et al, 1987). Identification of an OID for rHu-IFN-γ in patients with minimal tumor burden has lead to the development of clinical trials to test the hypothesis of whether the immunological enhancement induced by rHu-IFN-γ will result in prolongation of the disease-free and overall survival of patients in an adjuvant setting.

Two such Phase III trials are currently being conducted, one with melanoma patients following surgical excision of the primary lesion and the other in small cell lung cancer patients following induction of a complete response with chemotherapy and radiotherapy (Jaffe and Herberman, 1988). Both of these clinical trials are recruiting patient populations who face a high risk of recurrence following their primary therapy and who have no standard therapy available to reduce this risk. Further, these patients are immunologically competent compared to patients with advanced, clinically apparent, metastatic disease. Thus, they provide a near ideal population to test the hypothesis of an immunomodulatory mechanism of therapeutic efficacy. They may also be expected to benefit from such adjuvant immunotherapy.

In summary, the clinical strategy with rHu-IFN-γ is an example of the utility of a clinical hypothesis. First, preclinical information was derived to identify a cellular mechanism for the therapeutic activity, thereby providing a monitorable parameter which could be studied both preclinically and clinically. Second, clinical Phase 1b trials were initiated to determine the OID for the predictive effector cell parameter and third, appropriate prospectively randomized clinical trials initiated to test the hypothesis. We suggest that it is from this type of strategy that one can expect to further develop BRMs for the treatment of neoplastic disease.

Preclinical Development

The strategy for the identification of a practical clinical hypothesis is based on a systematic preclinical approach as summarized in Figure 1. Briefly, initial studies in normal animals are used to determine the pharmacology and pharmacokinetics of the immunomodulators. Based on these studies, the therapeutic properties of the cytokine are studied with some insight into a therapeutically active protocol. Following subsequent studies to determine the OTP (dose, route, schedule, sequence and timing and duration of cytokine administration), a series of protocol variations are utilized to determine the effector cells or mechanisms responsible for the therapeutic activity. Predicated on the OTP and the mechanism of therapeutic activity, clinical hypotheses can then be formulated and subsequently tested with some assurance of a rational rather than an empirical development. Subsequent strategies for the development/

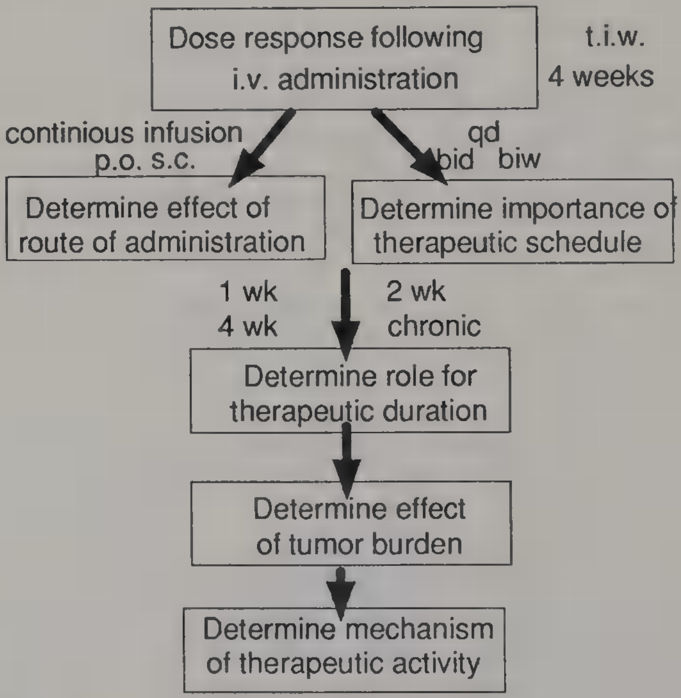

Figure 1.

identification of second generation proteins which address deficiencies of the initial lead agent may also be initiated in parallel to the initial clinical development.

Because metastasis is the major threat to cancer patients, the emphasis in the therapeutic evaluation of cytokines should be on the assessment of therapeutic efficacy against systemic lesions. Two models for the immunotherapy of metastasis are commonly utilized, one directed against tumor cells introduced in the host systemically by i.v. injection (experimental metastases) and the other, potentially more clinically relevant assessment of activity against metastatic foci which develop after the resection of a primary tumor (spontaneous metastases). In the spontaneous metastasis model, the host has been "conditioned" by tumor growth for some time prior to resection of the primary tumor at which time therapy is initiated. The spontaneous metastasis model is believed to be most predictive of systemic therapeutic efficacy in the clinical setting. Therapeutic activity demonstrated against established systemic disease in a relatively normal host (experimental metastases) or in a host conditioned by the growth of primary tumors with preexistent spontaneous metastases, however, may not be matched in the clinic. This difference may arise because the metastasis models utilize young, relatively healthy hosts bearing transplantable tumors. This situation may be different from that of a cancer patient, who may have been exposed to a carcinogenic insult many years before, and gradually developed progressive primary and metastatic tumor lesions over a prolonged period. Nonetheless, spontaneous metastasis model represents a more rigorous therapeutic model than prophylaxis or the treatment of intradermal, subcutaneous or peritoneal tumor models.

Initial information on the pharmacology, biodistribution, and pharmacokinetics can provide critical information for the design of therapeutic protocols (Talmadge et al, 1987; Shadduck et al, 1989). Parallel in vitro studies on the duration of receptor occupancy required for effector augmentation (Coffman et al, 1988) as well as the minimal dose required for maximal augmentation (Talmadge et al, 1987) when coupled with pharmacokinetic data on peak dose achieved, area under the curve and the beta half-life can provide critical information as to the route and schedule of administration. Thus, interleukin-2 requires receptor occupancy for 16 to 24 hours and approximately 100 units/ml for a maximal NK cell augmentation. To achieve these parameters clinically, either continuous IL-2 infusion, or multiple administration of high levels by i.v. or i.p. push are needed (Fig. 2). The initial clinical trials demonstrated that i.v. injection at such high levels results in appreciable toxicity (Moertel, 1986). It appears that a more pharmacologically appropriate route of administration is continuous infusion (Talmadge et al, 1987; Shadduck et al, 1989; Coffman et al, 1988; Moertel, 1986; Sosman et al, 1988) although this remains controversial (Alper, 1988).

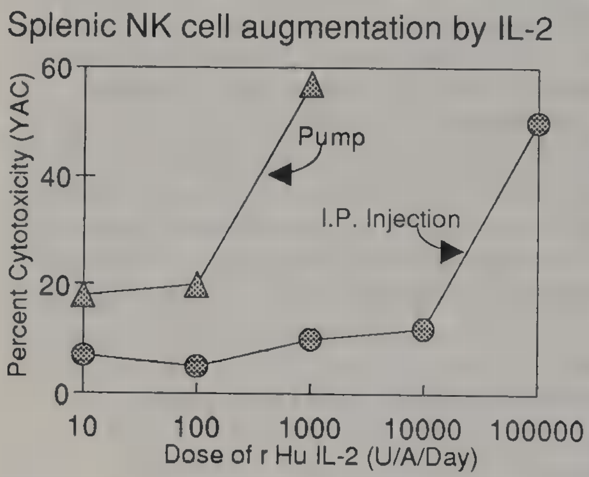

Fig. 2. A 7-day Alzet osmotic pump containing rH IL-2 or the excipient was implanted into the peritoneal cavity of mice. 3 days following pump insertion, spleen cells were obtained by normal methods and used in an NK cell assay. Age-matched groups of animals were also given injections of excipient or rH IL-2 at various doses by i.p. administration. 24 h later, the spleen cells were removed and the NK cell activity of these effector cells was determined. Previous studies demonstrated that 24 h following i.p. administration resulted in maximal NK cell activity.

Following the establishment of basic pharmacologic parameters, initial therapeutic studies are undertaken to determine a dose response. Once the dose response is identified the second objective is to address the importance of therapeutic schedule. This second phase study to determine the OTP has an experimental design such that the daily administration is biased towards lower, more easily tolerated doses, whereas a wider range of doses are tested by weekly or bi-weekly administration. If no therapeutic activity is observed with any type of schedule, no activity is observed following continuous infusion and a MTD is observed one can conclude that the agent has no therapeutic activity on the basis of either immunomodulation or direct chemotherapeutic activity. If, however, therapeutic activity is observed, the next objective is to determine whether an improved therapeutic index can be achieved predicated on the route of administration. Indeed, using the IL-2 clinical example, the lymphoid hyperplasia and therapeutic activity that are observed with continuous administration of high doses of rHu IL-2 suggest that *ex vivo* LAK cell induction, cultivation, and infusion technology may be unnecessary (Talmadge et al, 1987; Sosman et al, 1988; Sano et al, 1988; Thompson et al, 1988). Although clinical therapeutic activity and toxicology have been noted when rH IL-2 was administered by i.v. push with an aggressive dosage and schedule, most notably in combination with LAK cells or tumor infiltrating lymphocytes (Rosenberg et al, 1985; Topalian et al, 1988), the preclinical and clinical observation of therapeutic activity at low doses by continuous infusion suggest that rH IL-2 might be therapeutically effective using this less toxic protocol (Sosman et al, 1988; Alper, 1988; Sano et al, 1988; West et al, 1987).

Although, systemic disease is commonly treated by i.v. injection of cytokines; consideration should also be given to s.c. or i.m. administration, which may be beneficial because of a prolonged half-life of the agent in addition to the more convenient injection site (Maluish et al, 1988; Thompson et al, 1987). Protocols with subcutaneous or intramuscular administration also allow self-administration following a suitable training period. This is particularly attractive because of the perceived requirement for prolonged administration for maximal efficacy as discussed earlier and which can then be done on an out-patient basis.

The colony stimulating factors (CSFs), which are cytokines with "growth factor" like activity for bone marrow stem cells, have demonstrable and marked efficacy for decreasing the toxicity and potential for increasing the therapeutic index of chemotherapeutic agents and irradiation. The importance of chemotherapeutic drug dosage intensity, both preclinically and clinically in retrospective and prospective studies, has supported the hypothesis that the more aggressive use of chemotherapeutic agents also increases their therapeutic activity (Hryniuk and Bush, 1984; Tannock et al, 1988). Increased doses of chemotherapeutic agents can be achieved, in part, by preventing or decreasing the neutropenia associated with their aggressive use (Antman et al, 1988; Brandt et al, 1988). This goal can be achieved with autologous bone marrow transplants and/or the administration of agents which facilitate the outgrowth of stem cells and thereby reduce or prevent neutropenia. Autologous bone marrow transplants and accelerated reconstitution following myelosuppression are both achievable goals, which are facilitated with the use of the CSFs. The successful utilization of the CSFs is dependent on the schedule and timing of their administration. For example, if administered too soon after injection of a chemotherapeutic agent, that is, before the agent(s) have been completely metabolized, there is potential for increased toxicity. In contrast, if the agent is administered after complete metabolism of the agent, either alone or in conjunction with autologous bone marrow transplants, accelerated reconstitution and, in certain cases, a complete lack of neutropenia may be observed, at least in preclinical models.

The major opportunity is for the exploitation of cytokines with therapeutic advantages are likely to be identified in studies of combination treatments. Such studies can include the combination of cytokines with clinically efficacious cytotoxic agents or antibiotics (Mitchell et al, 1988), as briefly discussed above, as well as combinations of cytokines such as IFN-γ or IL-2 and TNF. Most recently a promising example of synergism has been suggested in AIDS to include AZT, rH-IFN-α and rH-CSF-gm. The objective is to determine the extent to which these agents might interact in a synergistic manner, with possible consequence to the reduction of tumor mass or virus titer. The combination of chemotherapy and immunotherapy may be active, via a number of mechanisms including: 1) reduction in tumor burden by cytoreductive agents resulting in a tumor burden more amenable to immunotherapy; 2) decreased suppressor cell activity resulting in an increased T cell adjuvant activity induced by agents such as rH IL-2; 3) the increased therapeutic activity of two cytoreductive - cytostatic modalities such as a chemotherapeutic agent and tumor necrosis factor or interferon; and 4) accelerated myeloid restoration due to increased stem cell activity thereby allowing more aggressive doses of chemotherapy and reduced neutropenia.

Identification of Therapeutic Mechanism

Identifying agents with therapeutic activity against established metastatic disease, optimizing the administration protocols, and understanding the mechanism of their therapeutic activity are the key checkpoints for the development of cytokines. The last objective of determining the mechanism of therapeutic activity is critical for initial clinical trials. Because immunotherapy does not directly kill the tumor cells with resultant rapid regression of the tumor lesions, but rather may slowly facilitate the regression of the tumor, it is unlikely that a Phase I or Phase II setting will be able to rapidly develop an agent predicated on therapeutic efficacy. Thus, it is particularly important, given the bell-shaped dose response curve of agents, that the optimal dose and protocol be determined prior to the establishment of clinical trials of efficiency. In order to optimize the clinical parameters, one needs to identify a parameter, preferably one in the peripheral blood which can be easily and reproducibly monitored and that is associated with therapeutic efficacy. Thus, one establishes prospectively randomized multiarm preclinical trials where the immune function is monitored at predetermined intervals and correlates effector cell function with appropriate parameters of therapeutic efficacy (survival). Typically, NK cell, LAK, CTL, macrophage, cytokine induction and subset analysis are monitored for such correlations. Obviously, a predisposing bias would be towards measuring effector function(s) in a technical manner that is feasible for clinical studies.

In conclusion, the utility of cytokines as a cancer therapeutic can be considered optimistically predicated on the progress to date. Although therapeutic activity has been found to date with cytokines in limited subsets of patients, which may be viewed as discouraging to some investigators, nonetheless, it should also be a stimulus to conduct careful basic and clinical experimentation aimed at obtaining further fundamental information on mechanism that would provide a basis for the successful clinical utilization of the cytokines.

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CELLULAR MATURATION AND ONCOGENE EXPRESSION DURING DRUG-INDUCED DIFFERENTIATION IN VITRO: A BRIEF REVIEW

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INTRODUCTION

The treatment of malignant disease with cancer chemotherapeutic agents generally relies on their capability for eradicating tumor cells, and cytotoxic drug therapy has led to significant progress in the control and cure of cancer. However, it is currently well recognized that new treatment modalities are required for further significant advances. One promising approach is based on accumulating experimental evidence that a diverse group of biological or chemical agents can promote cellular maturation and terminal differentiation of tumor cells (for recent reviews see Freshney, 1985; Bloch, 1986; Sartorelli et al., 1987; Marks et al., 1987; Sachs, 1987; Pierce et al., 1988; Hickman and Friedman, 1988). Such agents include hormones, growth factors and other regulatory peptides, polar compounds exemplified by dimethyl sulfoxide (DMSO), tumor promoters such as the phorbol ester tetradecanoyl phorbol acetate (TPA), and a number of clinically active anticancer drugs. Malignant cells have the capacity to differentiate spontaneously into benign cells in response to natural influences such as those found in the normal inductive embryonic environment, and malignant stem cells can be induced by various chemicals to terminally differentiate. Cancer is widely viewed as resulting from disordering of the normal controls of cell proliferation and differentiation. Even though cancer cells are probably genetically altered (Bishop, 1987), a variety of studies have demonstrated that such cells retain the potential to express a normal (or near-normal) differentiation program with consequent reduction or abrogation of malignant potential. Spontaneous differentiation of tumor cells has been observed both in vitro and in vivo. Even more provocative is the occasional report that tumor cells surviving chemotherapy appear to be phenotypically altered or differentiated.

Two major approaches have been exploited in experimental studies and are considered to hold promise for clinical application. One is the induction of terminal differentiation whereby tumor cells lose their proliferative capacity. The other is induction of differentiation whereby tumor cells concomitantly revert to a less malignant or nonmalignant state while retaining the ability to proliferate. The purpose of this brief article is to review recent progress in experimental studies of drug-induced differentiation and modulation of gene expression. A number of model systems have proven useful for such studies (reviewed by Freshney, 1985 and Reiss et al., 1986) and include (1) hematopoietic tumor cells such as mouse erythroleukemia (MEL) cells and human counterpart K562 cells as well as human myeloid leukemia HL-60 and ML-1 cell lines; (2) neuroectodermal tumor cells such as human or rodent neuroblastoma, pheochromocytoma, melanoma, and glioma cell lines; and (3) epithelial tumor cells such as the HCT-15 human colon adenocarcinoma, F9 mouse embryonal carcinoma, BeWo human choriocarcinoma, and SqCC/Y1 human epidermoid carcinoma cell lines. This review will focus on two model systems. One is the widely utilized HL-60 line which is unusually sensitive to the effects of differentiating agents. Since HL-60 cells can differentiate into granulocytes or monocyte/macrophage-like cells, depending on the chemical inducer, they represent an ideal system for investigations into regulatory mechanisms of lineage-specific maturation programs. The other model system is human neuroblastoma. A number of different human neuroblastoma cell lines are available and can be induced in vitro to differentiate into cells with morphological and biochemical properties of mature neurons or to transdifferentiate into cells with properties of other, nonneuronal neuroectodermal cell lineages. Since both the human leukemia HL-60 and most human neuroblastoma cell lines are characterized by amplification of the c-*myc* and N-*myc* oncogenes, respectively, studies of oncogene expression in cells induced to differentiate will also be described.

Finally, this review will summarize in vitro studies of cellular differentiation induced by cancer chemotherapeutic agents, with emphasis on natural product drugs. The capacity of sublethal concentrations of these agents to promote cellular maturation and alter expression of specific genes in experimental systems has provided the rationale for low dose therapy with anticancer drugs alone or in combination with differentiation or growth factors. In addition, alterations in differentiation or transformation phenotype associated with development of resistance to cancer chemotherapeutic agents will be discussed. While short-term exposure to natural product drugs such as actinomycin D or daunorubicin may induce differentiation of tumor cells, development of high levels of resistance may result in reverse transformation (Biedler et al., 1975). Preliminary studies of multidrug-resistant human neuroblastoma cells suggest that acquired resistance may be associated with alterations in either or both transformation and/or differentiation state (Meyers and Biedler, 1988).

HL-60 HUMAN MYELOBLASTIC LEUKEMIA CELL MODEL

The most widely studied model of drug-induced differentiation is the HL-60 cell line. It was established in cell culture from the peripheral blood of a patient with acute leukemia (Gallagher et al., 1979). The cells are commonly referred to as promyelocytes and exhibit properties of myeloid cells (reviewed by Collins, 1987). Recently the leukemia from which HL-60 was derived was reclassified (Dalton et al., 1988) as acute myeloblastic leukemia with maturation (FAB-M2) and reported to contain myeloblasts, promyelocytes, and a small proportion of more mature cells possibly reflecting the observed spontaneous differentiation of HL-60 cells in culture. The addition of any one of a wide variety of chemical agents to the culture medium promotes acquisition of properties of more mature cells. Depending on the agent used, HL-60 cells may manifest morphological, biochemical, cell surface, and functional characteristics of granulocytes, monocytes, macrophage-like cells, or eosinophils. While differentiated HL-60 cells may not represent exactly the normal counterpart cells and while the HL-60 line may not accurately represent myeloid leukemia, the extent and diversity of response to such a wide variety of inducing agents have made this line a model of choice for study of experimentally induced differentiation. The major pathways of HL-60 differentiation and some of the more commonly studied inducers are schematically outlined in Fig. 1.

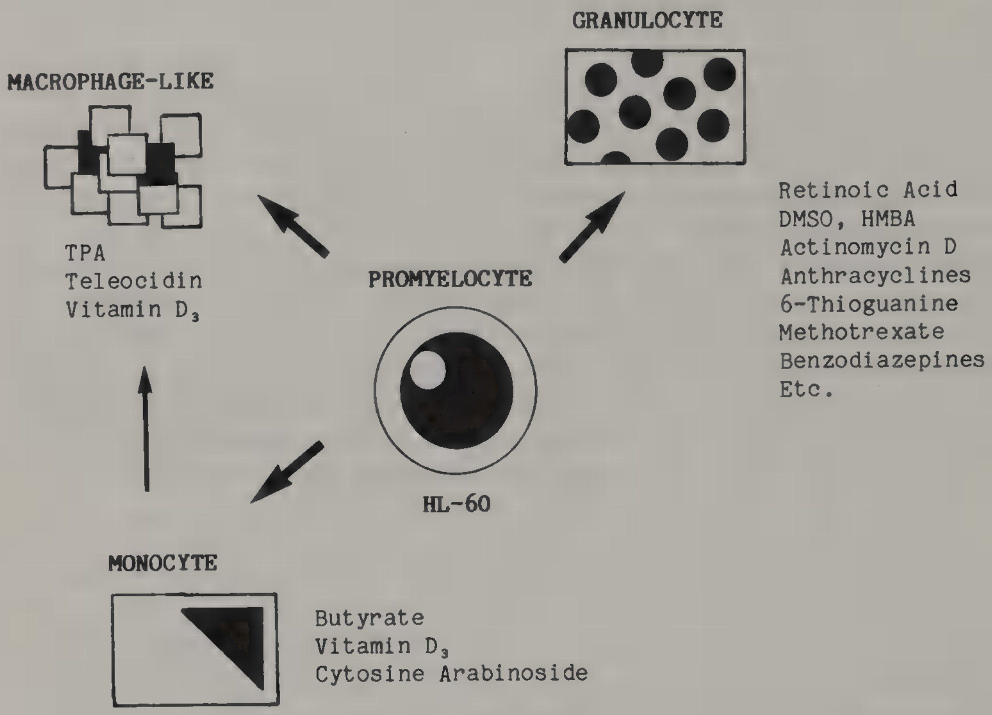

Figure 1. Schematic representation of induced myeloid differentiation in the human HL-60 promyelocytic leukemia cell line model system.

Part of the uniqueness of the HL-60 line is associated with its oncogene characteristics (Collins, 1987; Dalton et al., 1988). The c-*myc* gene is amplified and overexpressed about 15- to 30-fold. The cells also contain both a mutated N-*ras* and a mutated c-*neu* gene, and DNA from these cells has transforming activity for NIH-3T3 cells. Both p53 alleles are largely deleted and this gene is not expressed. In addition, one allele of the GM-CSF gene is truncated. Thus, the HL-60 system is a valuable albeit unique model for study of oncogene modulation during lineage-specific differentiation.

As indicated in Fig. 1, a variety of compounds including retinoic acid (RA), DMSO, and certain antimetabolites and natural product anticancer drugs induce HL-60 cells to differentiate into cells with morphological and functional features of mature granulocytes. Other compounds induce these cells to differentiate into cells with features of monocytes or macrophages. Although this review focuses on cancer chemotherapeutic agents, a considerable amount of background as well as new information has been derived with the use of other agents, particularly physiologic inducers, which are being widely used in the laboratory to distinguish between causal and consequential events in the differentiation process. For example, there have now been several reports that the vitamin D derivative, 1,25-dihydroxy vitamin D_3, is a steroid hormone that can regulate a variety of genes or gene products including cell surface differentiation antigens, oncogenes, and growth factors in addition to its classical role in ion homeostasis. The receptor for vitamin D_3 has been cloned; it is a member of the nuclear transacting receptor family that includes the estrogen, glucocorticoid, and retinoic acid receptors, among others.

CELLULAR DIFFERENTIATION INDUCED BY CANCER CHEMOTHERAPEUTIC AGENTS

As shown in Fig. 1, several conventional cancer chemotherapeutic agents induce differentiation of HL-60 to granulocyte-like or monocyte-like cells. A (non-comprehensive) review of experimental studies involving clinically useful cytotoxic agents reveals that many have been reported to induce differentiation in a variety of cell systems (Table 1). Among the natural product drugs, actinomycin D was substantially more effective in inducing morphological and functional differentiation of HL-60 than was adriamycin or daunorubicin. Interestingly, actinomycin D is an effective inducer for both human myeloid and mouse erythroid leukemia cells at similar concentrations (1-5 ng/ml). Moreover, these drugs may sensitize cells to respond to concentrations of differentiation-inducing factors (present in serum or in conditional medium) that would by themselves be ineffective (Honma et al., 1986). Schwartz and Sartorelli (1987) reported that the trisaccharide-containing antibiotic, marcellomycin, was an effective inducer of HL-60 granulocytic differentiation

TABLE 1. Cellular Differentiation Induced by Cancer Chemotherapeutic Agents: Experimental Studies

Chemical agent	Cell line	Reference
Actinomycin D	MEL mouse erythroid leukemia	Terada et al., 1978
	HL-60 human myeloid leukemia	Lotem and Sachs, 1980
	HL-60	Collins et al., 1980
	ML-1 human myeloblastic leukemia	Honma et al., 1986
Marcellomycin	HL-60	Schwartz and Sartorelli, 1982
Daunorubicin	ML-1	Honma et al., 1986
	M1 mouse myeloid leukemia	Kasukabe et al., 1987
Adriamycin	B16 mouse melanoma	Raz, 1982
	WEHI-3B D^+ mouse monomyelocytic leukemia	Gamba-Vitalo et al., 1987
Epirubicin	Human neuroblastoma	Rocchi et al., 1987
6-Thioguanine	MEL	Gusella and Housman, 1976
	HL-60	Ishiguro et al., 1984
Methotrexate	BeWo human choriocarcinoma	Friedman and Skehan, 1979
	HL-60	Bodner et al., 1981
Cytosine arabinoside	ML-1	Takeda et al., 1982
	HL-60 and U-937 monoblastic leukemia	Pinto et al., 1988
	G1-ME-N human neuroblastoma	Ponzoni et al., 1989
	Mouse neuroblastoma-glioma hybrid	Krystosek, 1989

whereas adriamycin was inactive. On the other hand, adriamycin was highly effective as an initiator of terminal differentiation of mouse monomyelocytic cells (Gamba-Vitalo et al., 1987). Decreased proliferation of treated cells was accompanied by a progressive increase in the proportion of differentiated granulocytic cells. As emphasized by Sartorelli and coworkers, the concentrations of anthracycline antibiotics that cause cytotoxicity are very close to those inducing differentiation, making it difficult to separate the two phenomena. Other studies involving natural product drugs are listed in Table 1. The anthracycline, epirubicin, has been reported to induce morphological and biochemical differentiation of five different human neuroblastoma cell lines, at a concentration corresponding to the lowest dose used clinically (Rocchi et al., 1987).

Certain of the earlier and several recent experimental studies utilizing the antimetabolites 6-thioguanine, cytosine arabinoside, and methotrexate are also listed in Table 1. Of particular pertinence to the concept of differentiation therapy are the findings of Ishiguro et al. (1984) that suggest that cellular differentiation induced by cancer chemotherapeutic agents reflects the interaction of multiple mechanisms, and not only their ability to inhibit DNA replication.

HUMAN NEUROBLASTOMA CELL DIFFERENTIATION MODEL

Human neuroblastoma is a tumor of early childhood and arises from the neural crest. Although initially responsive to chemotherapy, the tumor is ultimately fatal for about 80% of affected individuals, and the need for new treatment modalities is apparent. Maturation of malignant neuroblastoma to ganglioneuroblastomas or benign ganglioneuromas has been observed in both primary and metastatic tumors, either spontaneously or in response to treatment. Further, neuroblastoma has the highest overall rate of spontaneous regression of any malignancy. It has therefore been reasoned that agents capable of inducing cell differentiation under experimental conditions might be effective in the treatment of this tumor.

A substantial number of human neuroblastoma cell lines established in vitro and characterized biochemically (see

Biedler et al., 1983 for review) are available for studies of drug-induced differentiation. Malignant neuroblasts have properties of immature neurons, such as expression of neurotransmitter-synthesizing enzyme activities and presence of neurite-like cell processes. These cells, unlike mature post-mitotic neurons, are capable of continuous cell proliferation with only infrequent indication of spontaneous maturation to a less or nonproliferative state. A distinguishing feature of most well characterized human neuroblastoma cell lines, and of some 30% of tumors in vivo, is that they amplify and overexpress the protooncogene, N-myc. Amplification of this gene is associated with rapid tumor progression and a poor prognosis and may confer proliferative advantage for establishment of cells in continuous culture. The SK-N-SH line (Biedler et al., 1973), derived from a 2-year old patient with widely disseminated disease, is somewhat exceptional. These cells do not amplify N-myc (although they may exhibit low level expression), but contain an activated N-ras gene. Another feature of the SK-N-SH line, but not confined to this line alone, is the presence of (at least) two distinctive morphological cell types (Biedler et al., 1973; Ciccarone et al., 1979). One, termed N, is neuroblastic in its morphological and biochemical characteristics. The other, termed S for its substrate-adhesiveness, has properties of other neural crest-derived cells (Schwannian/glial, melanocytic, meningeal). Spontaneous interconversion between N and S cells in vitro has been well documented. Other human neuroblastoma lines can exhibit a similar transdifferentiation capacity (Ciccarone et al., 1989). Thus, the human neuroblastoma cell system, like the HL-60 system, provides a model for experimental study of lineage-specific cell differentiation, i.e., maturation to a more neuronal phenotype or transdifferentiation to another neural crest cell type.

A thrice-cloned subline, designated SH-SY5Y, isolated from the SK-N-SH line, has been used frequently in studies of induced differentiation (Table 2). Near-diploid SH-SY5Y cells are neuroblastic in appearance, have neurite-like processes, express catecholamine-synthesizing enzyme activities, and contain neurofilament proteins (Ciccarone et al., 1989). A variety of agents, exemplified by nerve growth factor (NGF), dibutyryl cAMP (dbcAMP), TPA, insulin, insulin-like growth factor II (IGF-II), and RA, have been shown to induce morphological and biochemical differentia-

TABLE 2. Induced Differentiation of Human Neuroblastoma SH-SY5Y Cells

Agent	Effect
NGF	Neurite extension; electrically excitable membranes (Perez-Polo et al., 1979; Spinelli et al., 1982)
dbcAMP	Neurite extension (Perez-Polo et al., 1979)
TPA	Neurite extension; occurrence of neurosecretory granules; lowered plasma membrane potential; increased noradrenaline content; higher neuron-specific enolase; appearance of Ca^{2+} channels; decreased muscarinic receptor activity (Påhlman et al., 1981; Spinelli et al., 1982; Åkerman et al., 1984; Heikkila et al., 1984; Påhlman et al., 1984)
Insulin, IGF-II	Neurite extension (Recio-Pinto and Ishii, 1984)
RA	Neurite extension; higher neuron-specific enolase (Påhlman et al., 1984)

tion (Table 2). The most striking and readily detectable cellular alteration is the extension of long neuritic processes as well as increase in number of processes per cell, with eventual formation of an extensive intercellular network. Additional characteristics of mature neuronal cells that have been observed in experimental studies of SH-SY5Y cells are listed in Table 2. Differentiation is accompanied by some degree of growth inhibition. Even though the same, clonally derived cell line was used in these studies, the various agents may have differential effects in terms of the phenotype induced. For example, Påhlman et al. (1984) assessed the effects of both TPA and RA. The cell processes induced by each of the agents were different in appearance, and at optimal concentrations TPA induced a 200-fold increase in noradrenaline whereas with RA the increase was only 4-fold. Increases in neuron-specific enolase were similar with both agents.

In a recent study, perhaps illustrative of the continued need to optimize generally effective experimental approaches, RA was utilized in combination with the benzoid antibiotic herbimycin A (Preis et al., 1988). Known to have antitumor activity, this compound had been shown to be growth-inhibiting and differentiation-inducing in other in vitro cell systems. In combination, the two agents induced neuronal differentiation (long neuritic processes) of SH-SY5Y, SK-N-SH, and Kelly cells to a much greater extent (60-81%) than did either agent alone. Furthermore, it was demonstrated in this study that RA by itself also induced phenotypic conversion or transdifferentiation of neuroblastic cells to nonneuronal, flat, S-type cells.

The ability of known differentiating agents to effect human neuroblastoma cell transdifferentiation has been observed by several investigative groups. The neuroblastoma cell lines SMS-KAN and SMS-KCNR established from two different patients showed morphological changes after treatment with either RA, 5-bromodeoxyuridine (BUdR) or dbcAMP (Reynolds and Maples, 1985). RA and dbcAMP induced neurite extension in most of the cells by day 2, whereas treatment with the thymidine analog resulted in a much slower time course of morphological alteration. Over a 14 to 30 day period, about 50% of cells developed a flat, S-type cell morphology and 50% showed neuronal maturation. The flat cells exhibited an altered antibody-binding profile resembling that of oligodendrocytes or Schwann cells in vitro. Sidell et al. (1986) reported that a neuroblastic subpopulation of SK-N-SH cells was transformed into large, flattened, substrate-adherent cells after exposure to RA for more than 7 days. In studies of human neuroblastoma GOTO cells, treatment with BUdR caused growth inhibition, conversion to morphologically flat cells, production of significantly large amounts of S100 protein and increased cyclic nucleotide phosphodiesterase (CNP) activity (Tsunamoto et al., 1988; Sugimoto et al., 1988). Since S100 protein and CNP are markers for glial and Schwann cells in the nervous system, these studies provide strong evidence that neuroblastic cells can be experimentally converted to other neuroectodermal cell types. Of four other human neuroblastoma cell lines tested, three showed no morphological changes in response to BUdR treatment whereas both flat and neuronal phenotypes were seen in the fourth (KP-N-RT) cell population. Another study tested the effects of RA and the ornithine decarboxylase inhibitor, α-difluoromethylornithine (DMFO), on the SK-N-BE(2) line (Melino et al., 1988). RA induced neuronal maturation whereas DMFO appeared to induce differentiation along another neuroectodermal pathway. These various studies indicate that differentiating agents are capable of inducing either neuronal maturation or transdifferentiation to another neural crest cell type, depending on the cell line/chemical inducer combination. The appearance of flat, S-type cells in response to differentiating agents suggests that these cells are a more mature cell type than the typical neuroblast. Further, the observations substantiate and extend the spontaneous N/S transdifferentiation phenomenon described by Ciccarone et al. (1989). Since it has been demonstrated in preliminary studies (Biedler et al., 1988) that flat S-type cells will neither grow in soft agar nor form tumors in nude mice, experimental induction of neuroblastoma cell transdifferentiation may have important implications for differentiation therapy of this disease. A number of different human neuroblastoma cell lines in addition to SK-N-SH and its clonally derived SH-SY5Y subline, as noted in the foregoing discussion of transdifferentiation, have been used to assess experimental induction of differentiation. All of the cell lines investigated, such as LA-N-1, LA-N-5, IMR-32, SMS-KAN, SMS-KCNR, and SK-N-BE(2), have elevated levels of N-myc mRNA and gene copy numbers ranging from 25- to 150-fold. For the most part, the agents used successfully to induce neuronal maturation of these various cell lines were dbcAMP, RA or a synthetic polyprenoic acid with properties of RA, or phorbol esters including TPA (Nishihara et al., 1981; Sidell, 1982; Spinelli et al., 1982; Haussler et al., 1983; Rupniak et al., 1984; Ohsawa et al., 1985; Sugimoto et al., 1987; Gotti et al., 1987). However, it was recently reported that epirubicin, an antitumor drug of the anthracycline antibiotic class, promoted morphological and biochemical differentiation of several different cell lines (Rocchi et al., 1987). These and other findings (e.g., Ponzoni et al., 1989) suggest that cancer chemotherapeutic agents that induce HL-60 cell differentiation (Table 1) may promote maturation of human neuroblastoma cells as well.

TRANSFORMATION/DIFFERENTIATION PHENOTYPE OF CELLS WITH ACQUIRED RESISTANCE TO CANCER CHEMOTHERAPEUTIC AGENTS

Since cells exposed for prolonged periods to cancer chemotherapeutic agents either in vitro or in vivo tend to develop resistance to those agents, it is of importance to know whether such cells are also resistant to agents capable of inducing cellular differentiation. Further, it can be asked whether drug-resistant cells may be phenotypically altered in ways that would be manifested as changes in proliferation, transformation, or differentiation potential.

Cells selected in vitro with any one of a large, diverse group of lipophilic natural product drugs, exemplified by the anthracycline antibiotics or the Vinca alkaloids, exhibit resistance to the selective agent and cross-resistance to others of the group. Such cells are termed multidrug-resistant. Earlier studies indicated that transformed Chinese hamster and mouse tumor cells selected in vitro for high levels of resistance to actinomycin D, daunorubicin, or vincristine underwent striking morphological changes toward phenotypically normal cells (Biedler et al., 1975). Assessment of tumorigenic capacity indicated that the multidrug-resistant cells were less malignant, i.e., were reverse transformed. Whether the cells were altered in their differentiative state could not be assessed because of lack of suitable markers. In current studies this question is being addressed primarily with the use of human neuroblastoma cells with acquired resistance to natural product drugs (Meyers and Biedler, 1988; LaQuaglia et al., 1989). A clonal derivative, BE(2)-C, of the SK-N-BE(2) line was used for independent selection of resistant sublines with actinomycin D, adriamycin, vincristine, or colchicine (Table 3). All of the sublines are multidrug-resistant in that they exhibit resistance or cross-resistance to all four agents and overproduce the characteristic high molecular weight plasma membrane glycoprotein, P-glycoprotein (Pgp), believed to act as a drug efflux pump. At this point, assessment of differentiative state of the resistant cells relies mainly on observation of cell morphology and estimates of N-*myc* expression.

An obvious consideration is whether any of the multidrug-resistant BE(2)-C sublines have acquired morphological characteristics of more mature neurons. Several of the sublines appear to have, on average, somewhat longer neuritic processes. However, it is difficult to distinguish between intrinsic features and morphological alterations induced by cytotoxic effects of drug during the

TABLE 3. N-*myc* Expression and Tumor-Producing Capacity of Multidrug-Resistant Human Tumor Cells Selected with Natural Product Drug

Cell line	Drug (μg/ml)	Resistance level	Pgp mRNA	N-*myc* mRNA	Tumorigenicity
Neuroblastoma					
BE(2)-C	none	1	±	+++	+++
BE(2)-C/ACT	0.2	167	+++	++	+
BE(2)-C/ACT	1	475	+++	+	+
BE(2)-C/ADR	5	104	++	+++	+++
BE(2)-C/ADR	20	100	ND	ND	+
BE(2)-C/VCR	10	567	++	+++	+
BE(2)-C/VCR	20	ND	++	+++	+
BE(2)-C/CHC	0.2	~40	+	++	+++
Colon adenocarcinoma					
S48-3s	none	1	–		++
S48-3s/ADR	10	~2,000	++		+

ACT, actinomycin D; ADR, adriamycin; VCR, vincristine; CHC, colchicine
ND, not determined

selection process. An inherent difficulty in this context is that substantial neurite extension is associated with substantially decreased proliferative capacity whereas development of drug resistance is dependent on selection of cells capable of continued proliferation. Other tumor cell types may be more amenable to assessment of morphological differentiation. For example, a 2000-fold adriamycin-resistant subline (Table 3) selected from a clone of SW-48 colon adenocarcinoma cells exhibits an increase in morphological differentiation compatible with continuous growth (Fig. 2).

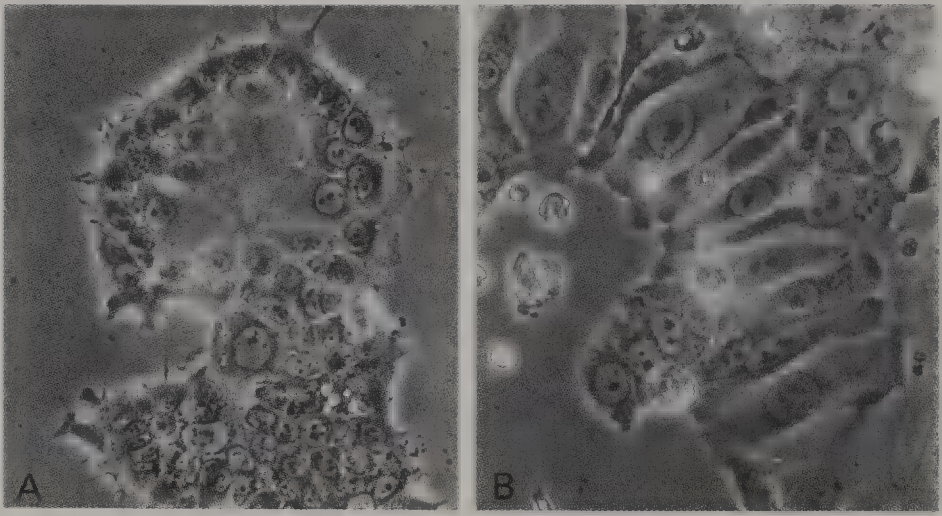

Figure 2. Phase contrast photomicrographs of clonal subline S48-3s (A), derived from SW-48, and its adriamycin-resistant subline (B). The morphologically heterogeneous resistant cell line comprises cells with a morphology and growth pattern resembling those of columnar epithelial cells, a cell type not observed in drug-sensitive cultures.

As discussed in detail in the next section, it appears that the N-myc oncogene is involved in the neuronal maturation of malignant neuroblastic cells. It has been found that the gene is expressed during normal embryogenesis, in early differentiative stages of some cell lineages in brain and kidney (Magrauer et al., 1988). BE(2)-C cells have a 150-fold increase in N-myc gene copy number and a high level of expression. Two of the four independently selected sublines show a 50% reduction in steady state level of

N-*myc* mRNA. Cells selected with the higher level of actinomycin D (1 µg/ml) have an even lower level of constitutive expression (Table 3, Fig. 3). These findings support the hypothesis of drug-induced differentiation accompanying multidrug resistance development. Ability of cells to grow in athymic (nude) mice and to grow in an anchorage-independent manner in soft agar are measures of cellular state of transformation. Results to date indicate a lack of correlation between N-*myc* expression level and transformation state (Table 3). From these ongoing studies, it is anticipated that further information will be gained about the role of N-*myc* in neuroblastic cells as related to cellular differentiation and to malignant potential.

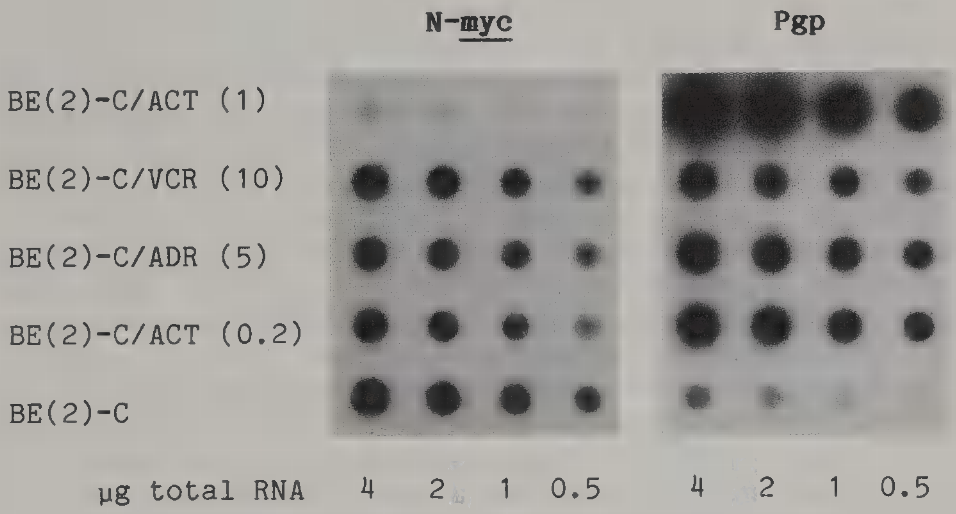

Figure 3. Dot blot analysis of steady state N-*myc* and P-glycoprotein (Pgp) levels in multidrug-resistant human neuroblastoma cells and controls. Total RNA was extracted from cells with guanidinium isothiocyanate/cesium chloride techniques, applied to nitrocellulose, and probed with appropriate ^{32}P-labeled cDNAs at high stringency. The BE(2)-C/ACT (1) cells tested contained little or no N-*myc* mRNA and a high level of Pgp mRNA, whereas parental BE(2)-C cells contain low levels of Pgp mRNA and high levels of N-*myc* mRNA.

MODULATION OF ONCOGENE EXPRESSION IN HUMAN NEUROBLASTOMA CELLS

Since most human neuroblastoma cell lines in culture are characterized by amplification of the N-*myc* proto-oncogene, this cell system provides a means of assessing whether oncogene expression is correlated with experimental induction of cell differentiation. N-*myc* encodes a nuclear protein and has been postulated to regulate gene expression. It is thus of potential importance to know whether this oncogene plays a role in normal or induced cellular differentiation. Thiele et al. (1985) reported that, in SMS-KCNR, SMS-SAN, and LA-N-5 cells (with 75-, 150-, and 25-fold amplification of the N-*myc* gene, respectively), a 5-hour exposure to RA resulted in an 85% decrease in steady state level of N-*myc* mRNA which preceded morphological differentiation. At the time of maximal differentiation, when cells had pronounced neuritic bundles, a 4- to 16-fold decrease was observed. Since N-*myc* expression decreased prior to onset of cell-cycle and morphological changes, it is possible that this gene is important in the implementation of a neuroblast differentiation program. In a separate study, Armatruda et al. (1985) found that RA induced a 75% decrease in N-*myc* expression in concert with morphological differentiation and inhibition of proliferation of LA-N-5 cells. In a recent, more extensive investigation of oncogene expression associated with RA-induced maturation of LA-N-5 and SMS-KCNR cells, it was found that, in addition to N-*myc*, the expression of c-*myc* and c-Ha-*ras* decreased prior to onset of morphological differentiation (Thiele et al., 1988a). These results are consistent with results of other recent studies indicating that cellular differentiation can be blocked by transfection and expression of c-*myb*, c-*myc*, or a mutant c-Ha-*ras* gene. It was also found that the decrease in N-*myc* expression during RA-induced differentiation is due to altered transcriptional regulation of the gene, with no significant difference in N-*myc* mRNA stability.

Regulation of expression of the c-*myb* proto-oncogene in human neuroblastoma cells has also been investigated (Thiele et al., 1988b). In normal tissues, expression of this gene is most prevalent in hematopoietic cell precursors, suggesting that it may play a developmental role in these cell types. However, in recent studies, c-*myb* expression has been detected in several different types of tumor cells, including those of neuroectodermal origin, suggesting that the gene may have a more general role in cell growth and differentiation. In RA-treated SMS-KCNR and LA-N-5 human neuroblastoma cells, c-myb mRNA was markedly decreased at a time when most cells had begun to extend neuritic cell processes. Change in expression was mediated by a decrease in rate of transcription. Thiele et al. (1988b) also examined c-myb expression in fetal and adult tissues. Higher expression levels were found in fetal as compared to adult cells, with the highest level in embryonic brain. Another gene whose expression is modulated during experimentally induced differentiation is that encoding the nuclear phosphoprotein, p53. The protein is thought to play an important role in the control of cellular proliferation, and there is a strong association between elevated levels of p53 and malignant transformation. Sidell and Koeffler (1988) recently reported that LA-N-5 neuroblastoma cells contain high levels of p53. RA-induced morphological and biochemical changes characteristic of neuronal maturation were accompanied by a marked decrease in levels of p53 protein and mRNA. However, the time-course of down-regulation, which did not occur until 48 hours after treatment and coincided closely with morphological differentiation, suggested that the p53 change did not trigger differentiation but instead reflected a secondary event.

SH-SY5Y cells, which as previously noted do not amplify the N-myc proto-oncogene, express this gene at detectable levels and exhibit an approximately 3-fold higher level of c-myc mRNA. Despite structural and functional similarities between these two cellular oncogenes, their possible interrelation and exact functions and modes of regulation are not known. It is thus of interest to compare their expression levels in response to treatment with chemical inducers of differentiation. In a study by Hammerling et al. (1987), after 24 hours of treatment with TPA, the expression level of c-myc but not N-myc was markedly reduced. After 4 days, when cells were overtly differentiated, N-myc mRNA levels were at 80% of initial values in contrast to <5% for c-myc. A somewhat different result was obtained with RA. A 4-day exposure resulted in a similarly decreased expression of both myc genes. N-myc transcript level was more markedly reduced with RA than with TPA. Of possible relevance to those observations is the earlier report (Påhlman et al., 1984) that the two inducing agents promote maturation, albeit neuronal in both cases, along apparently different pathways. One inference to be drawn from these studies is that neuronal differentiation of SH-SY5Y cells is compatible with substantial N-myc expression. Jalava et al. (1988) also observed a rapid decline in c-myc expression which preceded morphological changes and concluded that, for SH-SY5Y cells in particular, TPA-induced differentiation involves down-regulation of c-myc.

MODULATION OF ONCOGENE EXPRESSION IN HL-60 CELLS

Since HL-60 is an uncommitted precursor cell capable of undergoing either myeloid or monocytic/macrophage terminal differentiation and since it amplifies and overexpresses the c-myc proto-oncogene, it provides a useful model for studying the relationship of oncogene expression to normal and leukemia cell proliferation and differentiation. Studies of experimentally induced differentiation have also provided important insights into the interrelationship of several different oncogenes with maturation along different hematopoietic lineages. For example, exposure of HL-60 cells to either RA, DMSO, vitamin D_3, dbcAMP, hexamethylenebisacetamide (HMBA), or TPA resulted in a marked and rapid decrease in expression of c-myc or c-myb (Westin et al., 1982a,b; Reitsma et al., 1983; Watanabe et al., 1985; McCachren et al., 1986; Studinski and Brelvi, 1987). Cells acquired a granulocytic or monocytic/macrophage-like phenotype, depending on the inducing agent. Oncogene down-regulation was an early event and could occur within an hour of treatment whereas expression of a mature phenotype occurred after 24 or more hours, suggesting that changes in c-myc and c-myb transcript level are involved in the process by which differentiation is induced. Inhibition of HL-60 cell proliferation by itself does not result in a reduction of c-myc expression, again suggesting that it is differentiation rather than proliferation to which altered oncogene expression is related (Filmus and Buick, 1985).

Another gene involved in chemically induced HL-60 cell differentiation is the c-fms protooncogene. Expression of c-fms has been associated with differentiation of cells along the monocytic lineage. This gene is closely related to the receptor for macrophage colony-stimulating factor, CSF-1. In a number of studies it has been shown that maturation of HL-60 cells along the monocyte/macrophage pathway leads to a rapid increase in c-*fms* expression (Sariban et al., 1985; Studzinski and Brelvi, 1987; Imaizumi and Breitman, 1988). Not detected in uninduced cells, c-*fms* transcripts appear as early as 6 hours after TPA addition and are at maximal levels after 24-48 hours. A 20 minute treatment is sufficient for commitment along the monocytic differentiation pathway.

The HL-60 cell system has also provided information about the mechanisms that control down-regulation of c-*myc* mRNA during experimental induction of differentiation. It was earlier shown that a decrease in rate of transcription of the c-*myc* gene could account for the demonstrated decrease in transcript levels (Grosso and Pitot, 1985). In a more recent study, however, it was found that two distinguishable modes of regulation operate on c-*myc* during differentiation (Siebenlist et al., 1988). An increased blocking to elongation of nascent c-*myc* transcripts was demonstrated during the early phase of differentiation whereas, at a later phase, loss of transcriptional initiation was observed. In another approach, an antisense oligomer complementary to c-*myc* mRNA was used to inhibit c-*myc* expression (Holt et al., 1988). A decrease in c-*myc* mRNA, inhibition of growth, and myeloid differentiation ensued. Thus, direct inhibition of c-*myc* expression, without intervention of cell surface events, was responsible for differentiative and proliferative changes. In general, these various results support the hypothesis that c-*myc* drives cell proliferation whereas its absence allows differentiation.

SUMMARY AND OVERVIEW

This brief review of experimental induction of tumor cell differentiation in vitro is focused on several topics of biologic interest and potential clinical application. Areas that have been addressed and inferences that can be drawn are the following:

1. Malignant cells, even when genetically altered, have the capacity to mature to nonmalignant and/or postmitotic cells in response to a wide variety of inducing agents.

2. Whether cellular differentiation, under experimental conditions, will occur depends on both the particular tumor cell type and the particular inducing agent used. Whether differentiation of a tumor cell population will occur cannot, in general, be predicted prior to testing.

3. Both human myeloid HL-60 cells and human neuroblastoma cells are useful models for study of differentiation induction. HL-60 is unusually responsive to a wide variety of agents. In each system, the cells have the capacity to differentiate along more than one maturation pathway, thus providing an opportunity for studying lineage-specific differentiation in terms of both inducing agent and resulting cellular events.

4. As compared to hematopoietic cells, and with the possible exception of the human neuroblastoma cell system, there is a paucity of studies of differentiation induction in solid tumor cell types.

5. Experimental differentiation systems are useful for dissecting the role of oncogenes in cellular differentiation, proliferation, and/or transformation.

6. Cancer chemotherapeutic agents may themselves be effective experimental inducers of differentiation. Whether tumor cells which survive therapy in the patient are phenotypically altered in terms of differentiation or transformation needs continued assessment.

7. Whether cells developing resistance to cancer chemotherapeutic agents as a consequence of exposure to drug may have altered differentiation or transformation phenotypes is under investigation.

8. A promising but not yet extensively explored avenue of experimental investigation is the use of physiologic and other inducers of differentiation in combination with clinically effective cancer chemotherapeutic agents for optimization of results, i.e., to generate the largest proportion of (terminally) differentiated cells in the shortest period of time.

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MAMMARY GLAND AS A MODEL FOR STUDIES OF GENE EXPRESSION IN NORMAL AND MALIGNANT CELLS

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The study of the regulation of gene expression in cultured cells, particularly in epithelial cells, has both been hampered and facilitated by the loss of function that accompanies culture on traditional plastic substrata. Initially, investigations of differentiated function were made more difficult by the inadequacy of tissue culture methods developed to support growth of mesenchymal cells. However, with the recognition that the unit of function in higher organisms is larger than the cell itself, and that gene expression is dependent upon cell interactions with hormones, substrata, and other cells, came the understanding that the epithelial cell is profoundly influenced by its extracellular environment to express its phenotype. In the last decade research on epithelial cells has centered on culture conditions that recreate the appropriate environment for functional differentiation leading to promising and important results in many systems.

We use mammary epithelial cells from mid-pregnant mouse as a model to show that extracellular matrix (ECM) and some of its components (laminin, type IV collagen and heparan sulfate proteoglycan) play a fundamental role in regulation of milk protein synthesis and secretion. Using mouse c-DNA clones as well as polyclonal and monoclonal antibodies to the casein gene family (α, β and λ), transferrin, whey acidic protein and a human c-DNA clone to α-lactalbumin, we have shown that the extracellular matrix regulates milk proteins at multiple points:

a) mRNA level;

b) protein synthesis and accumulation;

c) secretory rate and route;

d) the level and the nature of proteolytic activities; and

e) hormonal inducibility;

The expression and secretion of whey acidic protein is additionally dependent upon the multicellular architectural restructuring which accompanies the onset of differentiation. We show further that changes in extracellular matrix could in turn induce changes in the level, the nature and the compartmentalization of extra cellular matrix components themselves. Thus, a complex interplay between hormones, ECM and "shape" determines whether or not a given protein is synthesized and secreted.

We conclude that:

1. The extracellular matrix on which the cells sit, is an extension of the cells and an active participant in the regulation of cellular function; i.e. the extra cellular matrix is an informational" entity in the sense that it receives, imparts and integrates structural and functional signals.

2 The ECM - induced functional differentiation in the mammary gland is mediated through changes in cell shape; i.e. that the structure is in large part "the message" to maintain differentiated gene expression.

3 The unit of function includes the cell plus its extracellular matrix; in a larger context, the unit is the organ itself.

These tenets and the data are consistent with a model of "Dynamic Reciprocity" (Bissell et al., 1982), where the extracellular matrix is postulated to exert an influence on gene expression via transmembrane proteins and cytoskeletal components. In turn, cytoskeletal association with polyribosomes is postulated to affect mRNA stability and rates of protein synthesis, while its interaction with the nuclear matrix could affect mRNA processing and, possibly, rate of transcription.

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LEAKY TUMOR VESSELS: CONSEQUENCES FOR TUMOR STROMA GENERATION AND FOR SOLID TUMOR THERAPY

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INTRODUCTION

Blood vessels are the conduits through which chemotherapeutic drugs, monoclonal antibodies, cytokines, and other therapeutic modalities reach tumors. If any of these agents is to be effective against solid or ascites tumors, they must have a means for exiting the vasculature and entering solid tissues and/or body cavities at tumor sites. Entrance into tumors or other tissues requires that circulating molecules pass through a succession of barriers, including the vessel wall and its underlying basal lamina, interstitial connective tissue, and the basal lamina that invests many epithelia, including tumors of epithelial origin. Passage of a therapeutic agent through each of these barriers may be dependent on either or both convection and diffusion and is regulated by a number of factors that relate both to the molecule itself and to tissue properties (Granger and Perry, 1983; Jain, 1987). Properties of the circulating agent that may affect its extravasation include intrinsic molecular characteristics such as size, shape and charge; binding, if any, to plasma proteins; and its respective concentrations in plasma and tissue compartments. Local tissue factors include properperties such as vascular permeability, hydraulic conductivity and reflectivity, the surface area available for intercompartmental exchange, and pressure gradients. Rate of local blood flow is also important in determining the availability of a circulating molecule over time for entrance into tumors, as compared with other organs, such as the kidney, that lead to excretion from the body.

We and others have established that the blood vessels that supply a wide variety of solid and ascites tumors are hyperpermeable or "leaky" to plasma solutes, and particularly to macromolecular solutes that are ordinarily retained within the vasculature (reviewed in Dvorak, 1986; Nagy et al, 1989). The finding that tumor blood vessels are hyperpermeable to macromolecular plasma solutes is significant for at least two reasons. First, extravasation of plasma fibrinogen and associated plasma clotting factors lead to local clotting and the formation of a cross-linked, extravascular fibrin-fibronectin gel that is deposited adjacent to tumor cells (Dvorak, 1986; Nagy et al,1989). This gel plays an important role in initial solid tumor organization; provides a matrix for tumor cell, inflammatory cell, and connective tissue cell migration; and serves as a provisional stroma that is subsequently replaced by vascular connective tissue. The analogies between tumor stroma generation and the process of ordinary wound healing have been rightly stressed in that both involve the replacement of a fibrin gel by vascularized connective tissue. Second, tumor vessel hyperpermeability has important implications for tumor therapy in that it predicts that molecules of large size (e.g.,monoclonal antibodies or small effector molecules bound to macromolecular carriers) will selectively concentrate in tumors vis-a-vis most normal tissues. In contrast, most small molecules (therefore, most commonly used cancer drugs) will not be selectively concentrated in tumors and will enter normal tissues as readily as tumors.

In this Chapter we will review the evidence for tumor blood vessel hyperpermeability, likely mechanisms responsible for this hyperpermeability, and some of the consequences of tumor vessel hyperpermeability for the design of new approaches to cancer treatment.

HYPERPERMEABILITY OF TUMOR BLOOD VESSELS TO MACROMOLECULES

The microvasculature supplying a wide variety of solid tumors in experimental animals exhibits a strikingly increased permeability to plasma proteins of varying size. Influx studies have demonstrated that radiolabeled fibrinogen, other plasma proteins, and tracers such as macromolecular fluoresceinated dextrans (FITC-D) enter solid tumors at a significantly faster rate (2 to ~ 17-fold faster, depending on the particular tumor and certain assumptions made in the calculation) than control tissues (Nagy et al, 1989). To put these data in perspective, the degree of hyperpermeability exhibited by vessels in solid tumors is comparable to that observed in healing skin wounds during the first few days after wounding (Brown et al,1988).

The extravasation of macromolecules from the plasma into the tissues is commonly limited by the degree of vascular permeability. However, other factors may also play a role and our influx data cannot be taken as a strict measure of increased peritoneal vascular permeability in ascites tumor animals. Also of importance is the capacity of tracer that has extravasated from blood vessels to pass through the interstitial space and, in the case of ascites tumors, to cross the mesothelial lining, a necessary step before molecules can enter the peritoneal cavity. As judged by fluorescence microscopy, the latter steps following vascular extravasation, are detectably slower for 5000 kD FITC-D than for FITC-D of smaller (e.g., 70 kD) size (J.A. Nagy and H.F. Dvorak, unpublished data). Thus, 5000 kD FITC-D remained localized to sites of vascular leakage for some time (e.g., 30 minutes) after extravasation, whereas FITC-D of smaller size rapidly diffused throughout the interstitium so that sites of vascular hyperpermeability rapidly became blurred and difficult to identify.

DISTRIBUTION AND TYPE OF LEAKY BLOOD VESSELS IN EXPERIMENTAL SOLID TUMORS

Recent studies of five experimental mouse and guinea pig tumors have sought to identify the vessels that leak in solid carcinomas (Dvorak et al,1988) (Fig. 1). Tracers of various types that could be followed in tissue sections by light or by

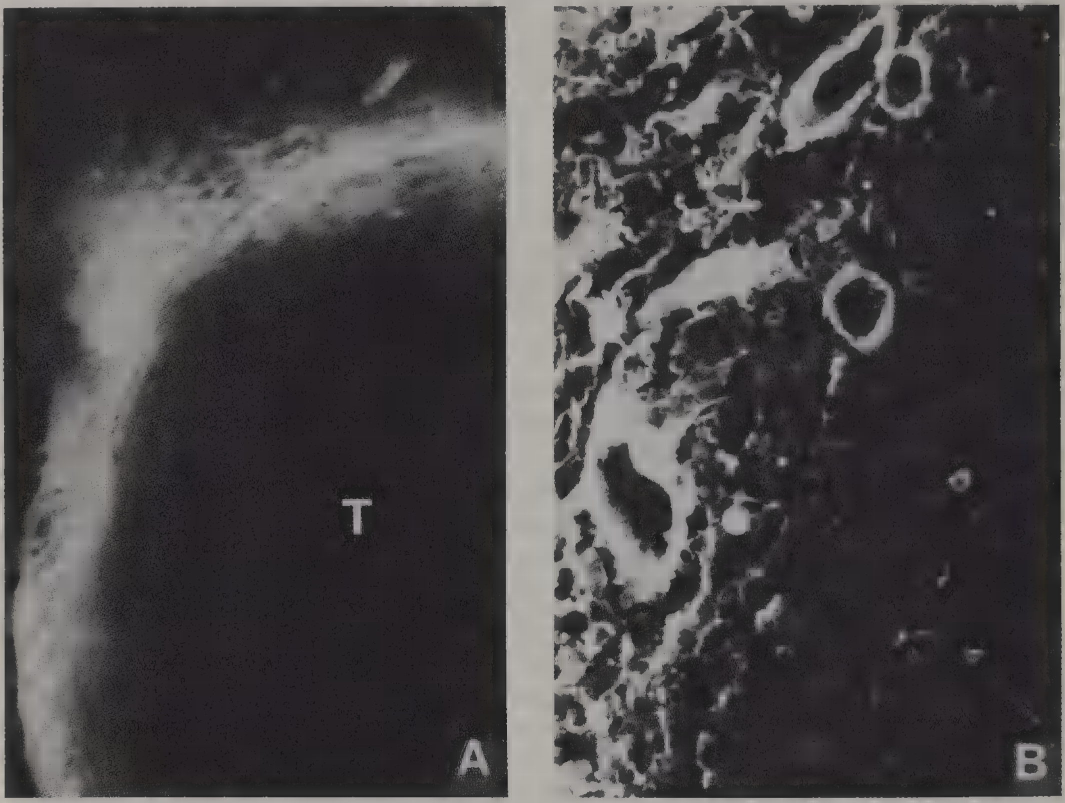

Figure 1. Extravasation of 150 kD FITC-D from the vessels enveloping a transplanted mouse Lewis lung carcinoma 15 min. after iv tracer injection. A. Extravasated FITC-D forms a bright fluorescent circumferential rim that surrounds but does not appreciably enter tumor (T). B. At higher power, leaky venules and small veins at the tumor-host interface are cuffed by leaked tracer (left). Again,little or no tracer extends among the tumor cells (right). A, x 30; B, x130. (B reprinted from Dvorak et al, 1988, with permission).

electron microscopy were injected intravenously at various intervals prior to sacrifice of tumor-bearing animals. Tracers included FITC-D of MW 3, 70 and 150 kD and colloidal carbon (corresponding mean diameters of ~ 2.4, 11.6, 17.4 and 50 nm, respectively). 3 kD FITC-D leaks extensively into the tissues from the capillaries of normal tissues, whereas the larger tracers ordinarily remain confined to the circulation with plasma half lives of > 4 hours. However, these same large tracers extravasated extensively in and about all five solid transplantable guinea pig and mouse carcinomas that we studied (Fig. 1). In all five tumors, leakage of macromolecular tracers (FITC-D of MW ≥ 70 kD and colloidal carbon) was confined to blood vessels in two locations: 1. vessels at the tumor-host interface and 2. vessels in the connective tissue surrounding and separating individual tumor nodules. In both locations, leaky vessels were confined to the connective tissue between and surrounding malignant cells and extravasated tracers did not come into intimate contact with tumor cells. This distribution of leaky vessels corresponds closely to that of fibrinogen leakage and extravascular clotting to fibrin that is also characteristic of these and other solid tumors.

In contrast, branch vessels that penetrated tumor nodules were not detectably hyperpermeable to these high molecular weight tracers. The failure to demonstrate leakage from tumor-penetrating vessels could reflect a failure of circulating tracers to enter them, perhaps because of sluggish blood flow, or, alternatively, could be attributed to characteristics of the endothelial lining that do not permit escape of intraluminal macromolecules. In favor of the first possibility, most penetrating vessels were engorged channels that contained macromolecular FITC-D at relatively low concentrations compared with comparably sized vessels at the tumor-host interface or in adjacent normal tissues. Nonetheless, intrinsic properties of penetrating vessels probably played a role in regulating tracer passage. The colloidal carbon and macromolecular dextrans that did enter penetrating vessels failed to leak, despite a prolonged opportunity to do so. In contrast, 3 kD FITC-D, which also entered penetrating vessels at low concentration, did leak from these vessels, suggesting that these vessels, like the normal capillaries of most tissues, were highly permeable to low molecular weight substances.

The nature of leaky tumor vessels was further defined by ultrastructural studies, making use of colloidal carbon as a tracer to identify hyperpermeable blood vessels. Colloidal carbon is particularly useful for this task in that it is readily visualized by electron microscopy, and, while able to penetrate the endothelial cells of hyperpermeable vessels, is largely trapped by the underlying vascular basal lamina where it remains for some time as a marker of leaky vessels.

Two important findings emerged from these studies. First, in all four tumors studied by electron microscopy, leaky vessels marked by subendothelial carbon deposits were found to be well differentiated venules and small veins; other types of vessel were not so labeled. Second, the leaky vessels of these tumors were lined by a continuous endothelium, similar to that found in most organs of the body including the adjacent normal connective tissue of the subcutaneous space. Therefore, the vascular endothelium of these tumors was not of the fenestrated or discontinuous (sinusoidal) types that are found normally in certain specific locations in the body (e.g., kidney, endocrine glands, liver, spleen) and that are known to be inherently hyperpermeable (Granger & Perry, 1983). We conclude that the hyperpermeability of these tumor vessels was not attributable to a change in intrinsic structural pattern but rather to a change in their functional properties.

Our ultrastructural studies were designed primarily to identify leaky tumor vessels, not to determine the mechanisms by which macromolecular tracers escaped from blood vessels. Therefore, tumors were harvested only at late intervals (e.g., 30 min.) after tracer injection, at times when most carbon had already been cleared from the circulation. Nonetheless, our studies did afford some insights into the mechanisms of tumor vessel hyperpermeabililty (Fig. 2). First, the hyperpermeability of tumor-associated vessels to colloidal carbon could not be attributed to vascular injury nor to structural defects of the vessel wall as may be found in newly formed blood vessels; damaged and structurally defective vessels were identified in tumors but rarely evidenced leakage as signified by the deposition of carbon in their walls. Second, vascular hyperpermeability apparently did not result from the formation of interendothelial cell gaps (Fig. 3). Gaps of this type form commonly in inflammation as a consequence

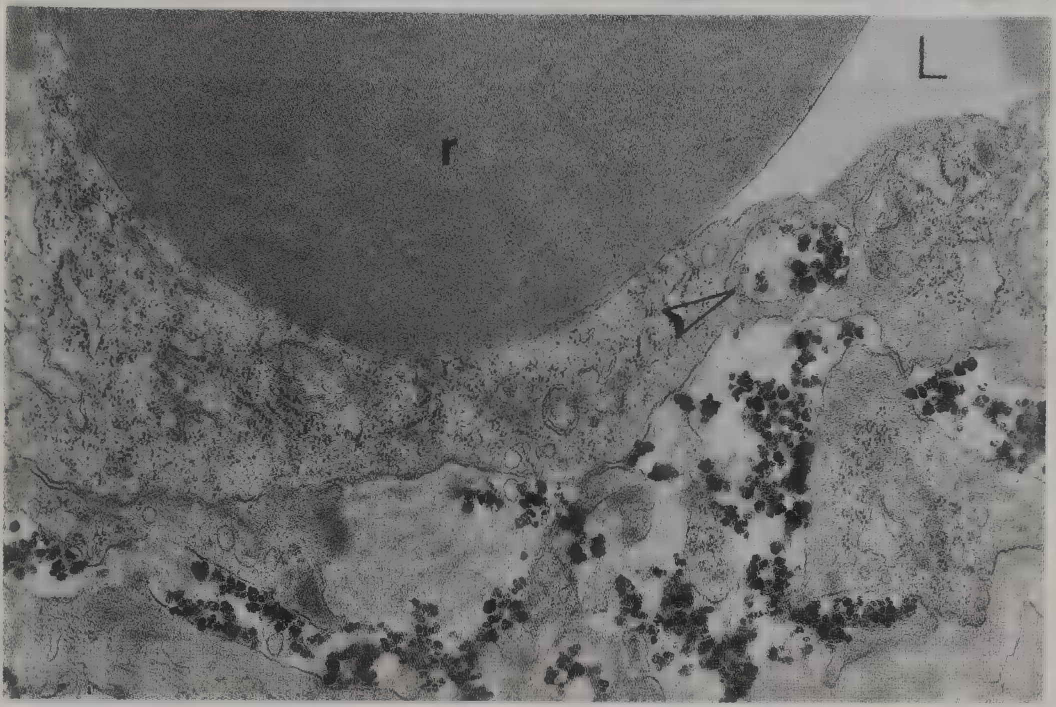

Figure 2. Electron micrograph of peripherally situated vessel adjacent to growing line 1 guinea pig bile duct carcinoma. Note extensive extravasated carbon in the basement membrane area. Endothelium (E) is undamaged. The vessel lumen (L) contains a red blood cell (r). A carbon-filled vacuole is present in the endothelial cytoplasm (arrowhead). x24,500. (Reprinted from Dvorak et al, 1988, with permission).

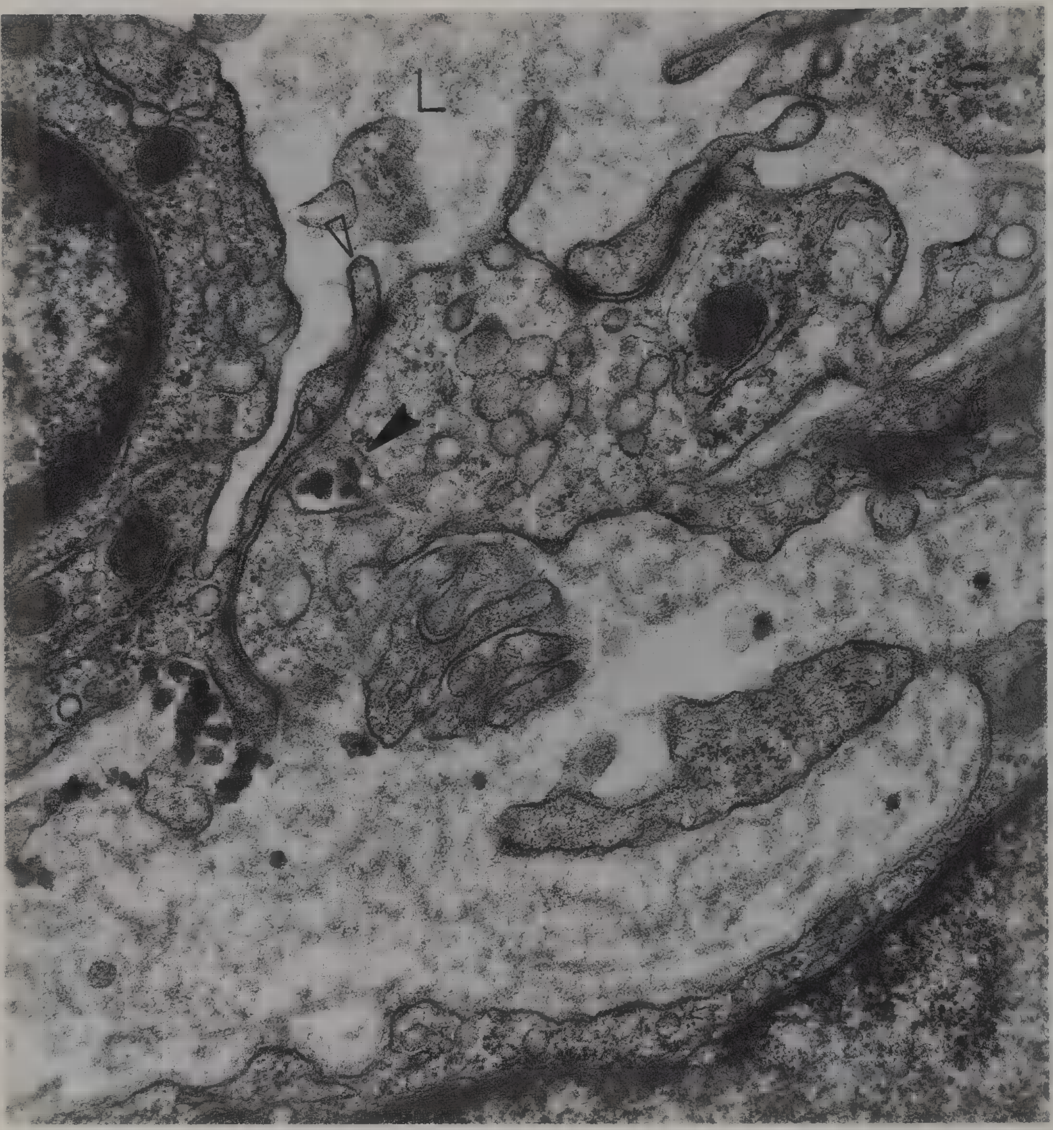

Figure 3. Closed interendothelial junctions in vessel adjacent to mouse ovarian tumor (MOT). Open arrowhead marks endothelial flap, closed arowhead a carbon-filled cytoplasmic vacuole. Carbon particles are also present in the edematous vascular basement membrane. L, lumen. x36,000. (Reprinted from Dvorak et al, 1988, with permission).

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of the contraction and pulling apart of individual endo-thelial cells in response to mediators such as histamine (Majno et al, 1969). However, open intercellular junctions were uncommonly observed in tumor vessels, including vessels that were manifestly leaky as judged by carbon labeling. If such gaps had been responsible for the hyperpermeability of peripheral tumor vessels, they would have been expected to be present commonly, continually, and without relation to tracer injection.

It seems apparent, therefore, that colloidal carbon (and, therefore, presumably other circulating macromolecules) exited from intact, well differentiated venules whose interendothelial cell junctions were closed. A possibility current-ly being explored is that these tracers traversed the endothelium of leaky vessels not by way of intercellular junctions but rather by way of vacuolar transport or by the formation of transendothelial cell channels. In support of this possibility, deep cytoplasmic clefts were found in endothelial cells of some leaky vessels, often adjacent to closed intercellular junctions. Carbon was also found in cytoplasmic vacuoles (Figs. 2,3), again often concentrated near interendothelial cell junctions. However, the late time of study, 30 minutes after intravenous tracer injection, precludes any firm conclusion. The question is currently being reinvestigated in tumors harvested within a few minutes of intravenous tracer injection at a time when tracer extravasation from leaky vessels may be expected to be maximal.

VASCULAR PERMEABILITY FACTOR (VPF) - A POTENTIAL MEDIATOR OF TUMOR VESSEL HYPERPERMEABILITY

Some years ago, we postulated that the hypermeability of tumor vessels might be attributable to a tumor-secreted mediator (Dvorak et al, 1979). That only mature veins and venules gave evidence of increased macromolecular transport was consistent with this mechanism; namely, that a tumor-secreted mediator interacted with specific cell surface receptors that were largely confined to differentiated venous or venular endothelial cells. Indeed, we have now demonstrated that a broad spectrum of animal and human tumor cells secrete such a factor which we have designated vascular permeability factor or VPF (Senger et al, 1983; Senger et al, 1986). Recently, VPF has been purified some 10-20,000-fold to homogeneity from serum-free guinea pig tumor cell culture medium (Senger et al, 1987). It is a protein of Mr 34,000-43,000 that is active at picogram levels and that, on a molar basis, is more than 10,000 times as active as histamine in enhancing microvascular permeability. Homology searches have demonstrated no identity or close similarity between the N-terminal amino acid sequence of VPF and database sequences, indicating that VPF is distinct from other proteins for which sequence data are available. Concentrations of VPF are particularly high in tumor ascites, suggesting that it has an important role in malignant fluid accumulation in body cavities as well as in solid tumors.

The mechanism of action of VPF is as yet poorly understood. Apparently, VPF does not act by releasing mediators such as histamine from basophils or mast cells or by way of other classic inducers of increased vascular permeability such as kinins or products of arachidonic acid metabolism. At present, a specific rabbit antibody is the only known inhibitor of VPF. Like histamine, VPF has been shown to induce reversible contractile separation of the endothelial cells that line venules and small veins without injuring these cells (Senger et al, 1983). Paradoxically, however, the leaky veins and venules in the several transplantable carcinomas we studied generally showed no ultrastructural evidence of interendothelial cell gap formation. Rather, endothelial junctions remained tightly closed. Therefore, leakage of tracers such as colloidal carbon apparently took place by some other mechanism, such as by vacuolar transport or by formation of transendothelial channels (see above). Further studies will be required to determine whether VPF is responsible for the increased vascular permeability that takes place by such mechanisms.

Other mediators have been claimed to be responsible for the increased permeability of the tumor vasculature (Greenbaum, 1979; Lobb et al, 1985; Matsumura et al, 1988), but the experimental basis for these claims appears incomplete.

CONSEQUENCES OF INCREASED PERMEABILITY OF TUMOR VESSELS

As noted earlier, tumor vessel hyperpermeability leads to the extravasation of plasma fibrinogen and thus to extravascular clotting, organization of tumor cells into a coherent mass, angiogenesis and stroma formation. Another potentially important consequence relates to tumor therapy. Our data suggest the preferential delivery to tumors of circulating macromolecules. Small molecules, such as most chemotherapeutic drugs, pass rapidly through the walls of normal small blood vessels (vessels of the central nervous system are an exception). Extravasation of these molecules is limited largely by blood flow; hence, their access to tumors is apt to be less dramatically enhanced by the increased vascular permeability exhibited by tumor vessels. If these data can be generalized to autochthonous human tumors, they would suggest that low molecular weight cytotoxic drugs would enjoy more favorable pharmacokinetics if they were attached to macromolecular carriers. In fact, it it likely that tumor vessel hyperpermeability to macromolecules is largely responsible for such success as has been observed with tumor imaging and therapy by monoclonal antibodies. Others have also suggested the use of macromolecular carriers for anti-cancer drugs (Schacht et al, 1985; Pastan et al, 1986; Matsumura & Maeda, 1986; Rodwell, 1988).

To be effective, cytotoxic agents must not only exit from tumor vessels but need also to diffuse through the interstitium to reach tumor cell targets. Our experience suggests that extravasated macromolecular dextrans enter solid tumors poorly as compared with the surrounding normal tissues (Fig. 1). On the other hand, Jain (1987) has reported, on the basis of an ear chamber model, that the interstitial space of solid tumors is characterized by high rates of flow and by high effective diffusion coefficients for macromolecules; together, these factors should favor interstitial transport of extravasated macromolecules (such as monoclonal antibodies) and their delivery to the proximity of tumor cells. A great deal more needs to be learned about tumor vessel permeability and the properties of the tumor interstitium to resolve this discrepancy. It is clear that studies of tumor vessel permeability and the convection and diffusion of extravasated macromolecules in the tumor interstitium will be critical to the burgeoning fields of imaging and therapy with monoclonal antibodies and to proposed treatments with monokines and other macromolecular biological response modifiers.

SUMMARY AND CONCLUSIONS

Our results may be summarized as follows: 1) The tumor microvasculature is hyperpermeable to macromolecular tracers, as compared with the vessels supplying many normal tissues. 2) In the case of the several solid transplantable animal tumors studied, leaky vessels are well-differentiated venules and small veins, lined by a continuous endothelium with mostly closed interendothelial junctions. Leaky vessels are concentrated at the tumor-host interface and in bands of stroma interspersed between tumor nodules; in contrast, tumor-penetrating vessels exhibit little or no leakage of macromolecules. 3) The mechanisms of vascular leakage have yet to be established but apparently cannot be attributed to vessel immaturity, structural defects, interendothelial cell gaps, vessel injury or development of a specialized (e.g., fenestrated or sinusoidal) endothelium. 4) Macromolecules that extravasate from leaky vessels also accumulate in tumors at higher concentrations than in the blood or control tissues. Increased tracer accumulation in tumors is attributable to increased tracer influx and possibly also to reduced tracer clearance (perhaps resulting from the absence or obstruction of lymphatics). However, the high molecular weight FITC-D tracers that extravasate into solid tumors remain largely confined to tumor stroma, do not extend far from the vessels from which they leaked, and make little contact with malignant cells. 5) In contrast, smaller tracers (e.g., 3 kD FITC-D) extravasate from tumor-penetrating vessels, from the capillaries of normal tissues, as well as from hyperpermeable vessels, and, unlike larger FITC-D, diffuse readily throughout solid tumors.

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EMBRYONIC MECHANISMS FOR ABROGATING THE MALIGNANCY OF CANCER CELLS

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THE EMBRYO CONVERTS MALIGNANT CELLS INTO NON-MALIGNANT CELLS

In 1974, Brinster reported that the mouse blastocyst could restore a normal phenotype to an embryonal carcinoma cell injected into it, and the cancer-derived normal cells under developmental control cooperated with their normal embryonic counterparts to eventuate chimeric offspring (Brinster, 1974). Chimerism was evidenced visually in the coat by patches of black color derived from genes carried by the cancer cell in contrast to the white coat color derived from genes carried by the blastocyst. Such chimeric offspring were usually chimeric in their internal tissues as well, such as in liver, gut, muscle and brain, as evidenced by glucose phosphate isomerase isoenzyme profiles (Bradley, 1987). The presence of cancer-derived somatic tissue suggested that the mechanisms which control the differentiation of the inner cell mass may also restore the normal phenotype to embryonal carcinoma cells.

We have found that abrogation of malignancy of embryonal carcinoma cells is the first event in their normalization and can be detected after only 4 hours of incubation in the microenvironment of the blastocyst (Pierce, et al., 1984). This observation provided the basis for a rapid assay with which to identify the mechanism of abrogation of malignancy. The degree of abrogation of malignancy can be measured by the decrease in frequency of clonal colony formation after exposure of the embryonal carcinoma cells to the blastocyst's microenvironment (Wells, 1982).

In this assay, almost all embryonal carcinoma cell lines were regulated by the blastocyst; in contrast, leukemia and sarcoma cells were not regulated (Table 1) (table compiled from refs. Pierce et al., 1984, Pierce et al. 1982, Pierce et al., 1989).

Table 1. Specific regulation of embryonal carcinoma cells by the blastocyst.

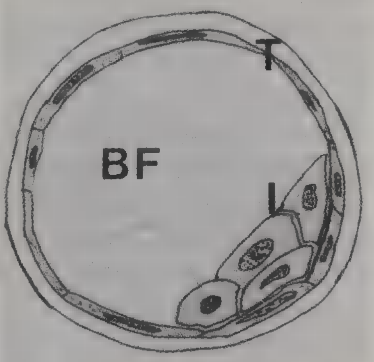
	# CLONAL COLONIES/ # OF ATTEMPTS	
TUMOR	EXPERIMENTAL	CONTROL
ECa 247	14/149 (9%)	68/160 (43%)
ECa P19	8/51 (16%)	110/240 (46%)
ECa PCC4	16/96 (17%)	230/402 (57%)
ECa 402Ax	21/93 (23%)	37/93 (40%)
ECa F9	43/123 (35%)	49/147 (33%)
Sarcoma 180	16/70 (23%)	16/80 (20%)
L1210 leukemia	20/70 (29%)	29/85 (34%)

This observation that the blastocyst can only regulate the cancer derived from its normal cellular population (inner cell mass _in vitro_ will spontaneously produce embryonal carcinoma but not leukemia and sarcoma) (Martin and Lock, 1983) suggested the idea of a embryonic regulatory "field", a temporal and anatomical site in the embryo which abrogates the malignancy of cancers derived from that field's normal lineages. For example, it was predicted that embryonic skin at the developmental time when it normally receives highly invasive melanoblasts from the neural crest should regulate melanoma and the embryonic breast primordium should regulate mammary carcinoma. These predictions have been verified experimentally: embryonic skin abrogates the malignancy of B16 melanoma cells but only at the developmental time when it normally receives melanoblasts (Gerschenson, et al. 1986); embryonic breast primordium abrogates the malignancy of mammary adenocarcinoma (unpublished data); neural crest derivatives such as somites abrogate the malignancy of C1300 neuroblastoma cells (Wells and Miotto, 1986); and the 10 day placenta abrogates the malignancy of myeloid leukemic cells (Gootwine, et al., 1982). Mice chimeric in their granulocytic tissues were obtained from embryos injected with the myeloid leukemic cells (Gootwine, et al., 1982), thus extending the concept that the embryo has mechanisms for converting cancer cells into normal cells under developmental control. The fates of implanted melanoma, mammary carcinoma, and neuroblastoma cells remain undetermined, but two possibilities exist: they could be converted into non-malignant, somatic cells as are embryonal carcinoma and myeloid leukemic cells or they could be killed (see section below on the cytotoxic activity in blastocele fluid).

CONVERSION OF EMBRYONAL CARCINOMA CELLS INTO NON-MALIGNANT CELLS REQUIRES SIMULTANEOUS CELL CONTACT AND EXPOSURE TO SOLUBLE FACTORS IN BLASTOCELE FLUID

We have chosen two embryonal carcinoma cell lines, ECa 247 and P19, for the investigation of the mechanisms of regulation in the blastocyst. They were chosen because they express very different developmental potentials after implantation into the blastocyst. P19 forms mid-term embryonic chimeras with a 60% frequency, but for reasons unknown none of these chimeras survive to term (Rossant and McBurney, 1983). Nevertheless, the P19 cells are converted into inner cell mass cells which participate in germ layer formation and development and which are clearly benign but probably abnormal. In contrast, ECa 247 does not form chimeric embryos; instead it differentiates into trophectoderm and presumably participates in the development of the extraembryonic tissues including the placenta (Pierce, et al. 1987).

Each component of the blastocyst--blastocele fluid (BF), inner cell mass (I), or trophectoderm (T)--was analyzed singly for its ability to convert ECa 247 cells to a non-malignant phenotype. None of these components alone converted ECa 247 into non-malignant cells, although a toxic activity was detected in blastocele fluid which killed 44% of the injected ECa 247 cells (see the following section)(Pierce, et al. 1989).



Our next step was to test various combinations of blastocyst components (Pierce, et al. 1989). Trophectodermal vesicles regulated both ECa 247 and P19; the vesicles were made by implanting two cancer cells at the end opposite the inner cell mass and then amputating and sealing the end containing the cancer cells bathed in blastocele fluid. Next, combinations of tumor cells and/or ICM cells were placed in an evacuated zona pellucida (as a carrier to prevent tumor cell contact with any cells other than those co-implanted into the zona carrier) which was then placed into the blastocele cavity of a giant blastocyst (made by fusing 8 eight-cell morulae). Contact with inner cell mass cells during exposure to blastocele fluid regulated only ECa 247 but not P19.

It was interesting that conversion of cancer cells to non-malignant ones required intercellular contact during exposure to soluble factors in blastocele fluid just as normal mechanisms of germ layer differentiation have also been reported to require cell-cell contact and soluble factors. Soluble bFGF caused differentiation of ectoderm into mesoderm in Xenopus blastulae (the equivalent embryonic structure to the mammalian blastocyst), an induction potentiated in a dose-dependent manner by TGF-B (Kimelman and Kirschner, 1987). However, a paper presented at the 1989 Meeting of the American Society for Cell Biology showed that bFGF did not induce mesoderm if the target cells were dissociated before treatment (Kimelman and Kirschner, 1989). Thus, soluble factors only induced differentiation of germ layers if there was proper intercellular contact between the target cells. These observations suggested a relationship between mechanisms of germ layer differentiation and cancer cell conversion.

The precise chemical nature of the required cell contact remains unknown. It may involve cell associated extracellular matrix molecules such as laminin and fibronectin. These molecules appear in the blastocyst before differentiation of the inner cell mass (Sanders, 1986). Furthermore, both fibronectin and laminin can determine the phenotype of differentiated embryonal carcinoma cells in vitro. For example, ECa 1003 cells grown in a serum-free medium on fibronectin coated plates terminally differentiate into neurons; if laminin coated plates are used, the cells differentiate into large patches of myotubes as well (Darmon, 1983). In addition, fibronectin promotes differentiation of ECa OC-15S1 cells even in serum-supplemented medium (Rizzino, 1983). Alternatively, a cell contact requirement may indicate the necessity of gap junctional communication between the normal cells and embryonal carcinoma cells. In several systems, gap junctional communication has been implicated in the control of cancer cells by surrounding normal cells (see the paper by Dr. Bertram, this volume). It is also quite conceivable that cell-cell contact may provide exposure to cell-associated or plasma-membrane bound growth factors (see paper by Dr. Derynck, this volume).

A CYTOTOXIC ACTIVITY IN BLASTOCELE FLUID MASQUERADING AS AN INHIBITOR OF PROLIFERATION

During experiments to determine the effects of blastocele fluid alone on embryonal carcinoma cells, an apparent growth inhibitory activity specific for ECa 247 and not for P19 was detected. For example, three ECa 247 cells placed in zonae pellucidae carriers and then into culture medium (negative control) proliferated to six to seven cells after 24 hours. After 24 hours in blastocele fluid three cells produced only three to four cells. In contrast, three P19 cells produced six to seven cells after 24 hours regardless of exposure to blastocele fluid. It was therefore concluded that blastocele fluid contained a specific inhibitor of proliferation of ECa 247 cells (unpublished).

However, dead ECa 247 cells were invariably observed in the zonae pellucidae carriers placed into giant blastocysts. To determine if the apparent inhibition of proliferation was in fact the result of cell death, single cells were used instead of three cells as in the previous experiment. It was observed that 44% of the single ECa 247 cells died (apoptotic cell death, i.e., condensation of the nucleus and cytoplasm followed by nuclear fragmentation) (Pierce, et al. 1989). The surviving 56% proliferated at control rates. The survivors were not permanently resistant to the cytotoxicity because 44% of survivors were killed by a subsequent 24 hour exposure to blastocele fluid. The reason for this apparently selective killing remains unknown.

As demonstrated by this example, a cytotoxin, which killed some but not all of the cells in a population, easily masqueraded as an inhibitor of proliferation. Identification of an inhibitor of proliferation requires proof that it prolongs cell cycle time without affecting cell viability. One of the few studies of this kind has been reported by Shipley, et al., demonstrating that TGF-B prolongs cell cycle time in AKR-2B fibroblasts in vitro (Shipley, et al. 1985). A decrease in $[^3H]$-thymidine incorporation has been used as evidence for inhibition of proliferation. However, this result does not distinguish between a cytotoxin and a growth inhibitor, since dying cells would not synthesize DNA. Furthermore, the demonstration of a shorter population doubling time after removal of the factor ("reversibility") does not prove that the factor is non-cytotoxic because the proportion of cells in a population capable of cell division would increase after removal of a cytotoxin.

EXTRA- AND INTRACELLULAR POLYAMINES AND AMINE OXIDASES IN PROGRAMMED CELL DEATH ("APOPTOSIS")

It was of interest to identify the chemical nature of the cytotoxin in blastocele fluid: (i) dead cells appear in the inner cell mass during blastulation, so identification of this compound may shed light on mechanisms causing programmed cell death during embryogenesis, and (ii) the compound exhibits a high degree of selectivity (distinguishing embryonal carcinoma cell lines with different developmental potentials), so its structure and mechanism of action may reveal how to design cytotoxic therapeutic agents of higher therapeutic indices than those currently available.

An alternative source of blastocele fluid was needed for biochemical analyses because each blastocyst contained 1 nL of fluid. We discovered that ascites tumors of the teratocarcinoma C44 contained embryoid bodies which closely resembled blastocysts. Ultrastructural morphology and immunohistochemical staining patterns showed that these embryoid bodies contained all of the cell types found in blastocysts, albeit they were present in abnormal proportion. Most importantly, they abrogated colony formation of microinjected ECa 247 but not P19 cells, the characteristic selectivity of normal blastocele fluid. The neoplastic blastocele fluid was extruded from these embryoid bodies during a 5 minute ultracentrifugation at 450,000 xg. This procedure resulted in minimal contamination from cell associated molecules, since the flattened embryoid bodies re-expanded with fluid after 24 hours if placed back into culture medium (unpublished data).

The blastocele-like fluid isolated from C44 embryoid bodies was toxic to both ECa 247 and P19 in culture medium. Anionic but not cationic or hydrophobic solid phases adsorbed all detectable cytotoxic activity. This result suggested that the cytotoxins in the fluid might be related to polyamines--small, positively charged organic amines. Both spermidine and spermine but not putrescine were cytotoxic in the presence of bovine serum because of the presence of a serum enzyme named amine oxidase which catabolized spermidine and spermine to mono- and dialdehydes, respectively, with concomitant generation of H_2O_2 (Tabor, et al., 1955). The generated H_2O_2 killed the cells (Henle, et al., 1986). Thus the question was restated: were there cytotoxins in the blastocele-like fluid from C44 distinct from spermidine and/or spermine?

To answer this question, the fluid was assayed for cytotoxic activity in the presence of two enzyme inhibitors of serum amine oxidase, aminoguanidine and beta-mercaptoethanol. These inhibitors prevented killing of both ECa 247 and P19. This result indicated that extracellular polyamines were present in the blastocele-like fluid and therefore probably in normal blastocele fluid as well. It also indicated that the cytotoxicity due to the activity of serum amine oxidase prevented any measurement of the effects due to the extracellular polyamines in the blastocele-like fluid.

To study the effects of extracellular polyamines and simultaneously to eliminate the interference from serum amine oxidase without using enzyme inhibitors which might also inhibit cell-associated or intracellular amine oxidases important for selective cytotoxicity, we used an aromatic polyamine called Janus Green B, whose spatial arrangement of nitrogen atoms resembled a single, constrained conformation of the aminopropyl ends of spermine and spermidine and the diaminobutane moiety of putrescine (see accentuated bonds in the figure at right).

Janus Green B was not a substrate for serum amine oxidase because the terminal nitrogens were methylated and ethylated. Its inability to function as a substrate for serum amine oxidase was shown by an identical LD_{100} on B16 target cells cultured in serum free and serum supplemented medium.

$NH_2(CH_2)_4NH_2$

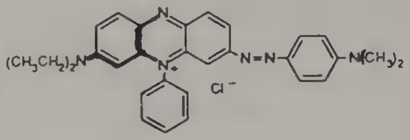
putrescine

$NH_2(CH_2)_3NH(CH_2)_4NH_2$

spermidine

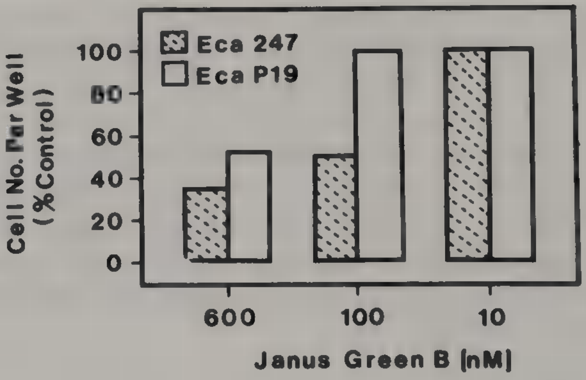
$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$

spermine



Janus Green B

Janus Green B, at 600 nM, caused death of both ECa 247 and P19. However, at concentrations around 100 nM, it caused death of 50% of ECa 247 cells but 0% of P19 cells.



Therefore, at some concentrations, a particular spatial arrangement of a structural motif found in polyamines exhibited the same cell-specific cytotoxicity as did blastocele fluid. Although structure-activity studies would be required to show that the spatial arrangement of the nitrogen atoms was responsible for the cytotoxic activity, the data were consistent with the idea that extracellular polyamines mediated the cytotoxicity in blastocele fluid. Janus Green B perhaps accessed intracellular amine oxidases or alternatively was metabolized by the target cell into a form that functioned as a substrate for intracellular enzyme and the generation of H_2O_2.

Apoptosis (programmed cell death) is not uncommon throughout the embryo, in tumors, and even in normal epithelium. Cell loss via apoptosis has been postulated to balance mitosis in these tissues (Kerr, et al., 1972). This type of cell death, which has been termed "death from within" or "suicide", occurs in rapidly proliferating populations of cells. Since these rapidly proliferating cells contain high intracellular levels of polyamines, we have hypothesized that cytoplasmic amine oxidases generate high levels of intracellular H_2O_2 during apoptosis and thereby kill the cell. Either induction of cytoplasmic amine oxidases or their relocation to the cytoplasm from another subcellular site could occur during the cell death program. Amine oxidases microinjected into a target cell have been reported to cause death from within (Bachrach, et al., 1988). Experiments are currently underway to determine the role of intracellular polyamines and amine oxidases during apoptotic and non-apoptotic cell death in the blastocyst.

In light of the extracellular polyamines discovered in the blastocele-like fluid of C44 embryoid bodies and therefore probably present in normal blastocele fluid as well, we have also hypothesized that mechanisms of cell death and tumor cell regulation in the blastocyst involve extracellular polyamines. Selective polyamine cytotoxicity could be accomplished via binding molecules which present extracellular polyamines in a fixed spatial configuration, such as that in Janus Green. Alternatively, these extracellular polyamines may serve as substrates for plasma membrane-bound, extracellular amine oxidases, whose cellular distribution produces the observed selectivity. ECa 247 but not P19 cells might have amine oxidases on their surfaces, thereby localizing the oxidation of extracellular polyamines and the formation of cytotoxic H_2O_2 to the immediate vicinity of the plasma membrane of the target cell.

IMPLICATIONS FOR TUMOR THERAPY, CONTROL OF TISSUE RENEWAL (HOMEOSTASIS), APOPTOTIC CELL DEATH, AND TUMOR PROGRESSION

The embryo is capable of abrogating the malignancy of five of five cancers if the proper embryonic field is utilized. Furthermore, in two of these systems, the embryo converts the microinjected cancer cells into cancer-derived, somatic tissues. The developmental potential of the injected cancer cells determines the phenotypes of the cancer-derived somatic cells. Three conclusions important for cancer therapeutics can be drawn from these results: (i) the embryo has mechanisms to eliminate the malignant phenotype; (ii) the resulting conversion of a malignant cell into a normal, homeostatically-controlled cell lasts into adulthood, presumably well after the conversion mechanisms are removed; and (iii) tumor cells do not necessarily "beget" tumor cells but rather have the potential to behave as normal somatic cells. Identification of the molecular mechanisms which underlie the abrogation of malignancy may eventually lead to an alternative form of cancer chemotherapy, the non-toxic conversion of cancer cells into benign somatic tissues.

The observation that the effects of cell death (by apoptosis) can be misinterpreted as prolongation of the cell cycle time suggests that cell death may mediate homeostasis in renewing tissues. In fact, several investigators have concluded that cell death by apoptosis is the major pathway of cell elimination in situ (Kerr, et al., 1972). A class of molecules called chalones has been postulated to regulate tissue renewal (Langen, 1985). They have been defined as non-cytotoxic, reversibly-acting, species non-specific inhibitors of cell proliferation which are tissue specific. Their release from terminally differentiated cells has been hypothesized to inhibit the proliferation of the stem cell compartment of that tissue. However, chalones may in fact be cytotoxins which kill some but not all of the stem cell population. Of particular pertinence to this paper is the inability to separate chalone activity from polyamine "contaminants" (Barfod, 1980). These polyamine "contaminants" may be the actual chalone molecules which exerts its effects through amine oxidase-dependent toxicity or perhaps through binding molecules which can orientate a polyamine into a cytotoxic configuration, such as that mimicked by Janus Green B.

Apoptosis, or programmed cell death, may be caused by the activities of amine oxidases upon polyamines and the consequent generation of H_2O_2. We and others (Agostinelli, et al., 1988) are currently exploring the possibility of targeting these pathways for tumor therapy. Because at least some tumor tissues contain extracellular polyamines, we have reasoned that administration of amine oxidases should cause tumor destruction. Once the initial H_2O_2 is generated in the tumor, a positive feedback reaction would be initiated in which cell death causes further release of polyamines into the extracellular space, which causes production of more H_2O_2, more cell death, and so on. Normal adult tissues should be spared because their polyamines for the most part would be intracellular.

As a final point, the C44 embryonal carcinoma line is derived from the same parental tumor, OTT6050, as is ECa 247 (Pierce, et al., 1987, Monzo et al., 1983). Since C44 under certain circumstances produces a cytotoxin for ECa 247, then the parental tumor can undergo "autoselection" as the C44 strain kills the strains with trophectodermal potential like ECa 247. Therefore, tumor progression is not necessarily due to selection by the host; it is also due to the effects of the soluble factors produced by one tumor subpopulation upon another. Tumors can be considered mosaics of different subpopulations in various patch sizes. The secretions and patch size of the subpopulations will determine the biology of each tumor and also the biology of the remaining tumor after selective removal of subpopulations. The ability of a large tumor to suppress the proliferation of a small distant tumor may reflect only tumor progression, the selective advantage of one subpopulation over another via the secretion of tissue-specific cytotoxins. It has been stated at this Symposium that tumors in animal model systems appear to be under self-generated homeostasis but that this homeostatic mechanism is different from that which operates in the corresponding normal adult tissue (see paper by Dr. Cameron, this volume). We would absolutely agree and would extend this concept by stating that the mechanisms for homeostatic control of tumor size, which probably involve cytotoxins that cause apoptosis, caricaturize the mechanisms which mediate homeostasis in the corresponding embryonic tissue from which the normal lineage of that tumor arose; these mechanisms are absent in the adult.

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The Chemoprevention of Human Cancer: An Overview

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INTRODUCTION

The enhanced life expectancy enjoyed by most individuals during this century has been achieved largely through disease prevention through the recognition of the etiologic agents responsible for many human diseases and the adoption of appropriate immunization and/or sanitary procedures. Only recently has the prevention of malignancy received much attention, principally through the institution of mass screening procedures. Success in this area is best exemplified by the dramatic decrease in cervical carcinoma incidence since the introduction of the Pap test (Cramer, 1983). An alternative approach to cancer prevention is the use of defined chemical agents to interfere with the carcinogenic process. This approach, termed chemoprevention (Bertram et al., 1987), relies on our enhanced understanding of cancer risk factors in general, and the mechanisms of carcinogenesis in particular, has many potential advantages over screening methods. For example, by the use of systemic administration, chemopreventive agents can reach anatomic sites not accessible by screening,furthermore, by preventing even the early pathological changes detected by current screening programs the need for invasive therapy and risk of therapy failure is much reduced. The logic behind the old adage "prevention is better than cure" appears irrefutable in this context.

What is the evidence that such an approach is feasible? Evidence stems from separate sources: in studies of the epidemiology of human cancer, certain dietary factors were found to be associated with a reduced risk of cancer; in laboratory studies of defined chemicals known to be components of diets so identified were shown to reduce the incidence of experimental carcinogenesis; finally in preliminary clinical studies, these chemicals have been successfully tested in high-risk individuals or in those with preneoplasia.

Epidemiologic studies, principally conducted in migrant populations, have led to the realization that the majority of human cancer in the most commonly involved organs is induced by exogenous agents rather than being of genetic origin (Doll and Peto, 1981). In concept at least these tumors are preventable. The major exogenous factors identified are, of course, tobacco and diet; together they are believed to contribute to about 70% of excess cancer mortality in the United States (Doll and Peto, 1981). Only a small proportion of cancers appear genetically linked of which bilateral retinoblastoma (Benedict et al., 1983) and colon carcinoma as a consequence of familial polyposis (Leppert et al., 1987) are two important examples. In these diseases, it appears that cells spontaneously exist in an early stage in the carcinogenic process, subsequent events required for the production of a neoplastic cell may then occur. While these secondary events may be rare on a per cell basis, the target cell population may be sufficiently large to make these events frequent at the level of the organ.

Steps in the Carcinogenic Process

Evidence for multiple steps in the carcinogenic process comes from animal studies, from studies of cultured mammalian cells and from studies in humans exposed to industrial carcinogens or nuclear radiation. The prolonged latency between exposure and clinical tumors in humans is entirely consistent with the data obtained in rodent models.

In order to illustrate the potential sites for active intervention, an overview of the carcinogenic process is presented diagramatically in Figure 1. As seen in this figure, carcinogenesis may be separated mechanistically and temporally, into three basic steps:

initiation, the formation of a genetically altered cell; promotion, the phenotypic expression of this damage; and progression, the clonal outgrowth of neoplastic cells with progressively more malignant properties.

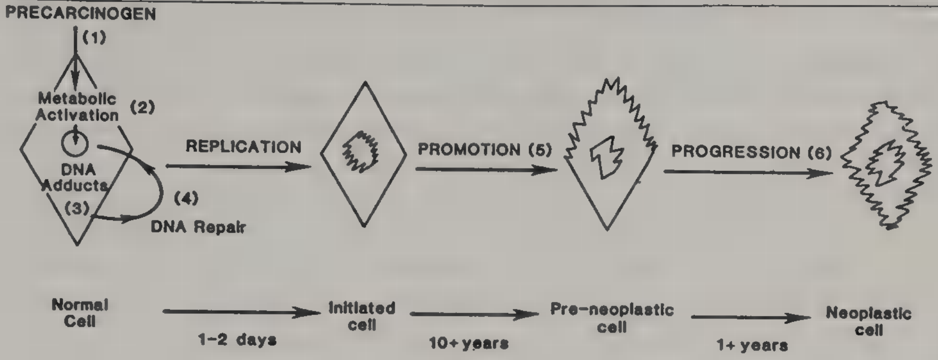


Figure 1. Steps in the chemical induction of neoplasia. Loci for intervention. Step 1: Avoidance of exposure is clearly the most desirable form of cancer prevention but is outside the scope of this paper. In the special case of endogenous formation of nitrosamines and of fecal mutagens, chemopreventive agents can block formation and presumably alter exposure. Step 2: Most chemical carcinogens require metabolic activation; chemoprevention can be achieved by blocking or diverting this metabolism towards less genotoxic products. Step 3: The proximate or ultimate carcinogenic form of a carcinogen may be intercepted prior to its reaction with DNA. Step 4: DNA replication past carcinogen-induced DNA lesions appears to be an important step converting chemical damage to genetic alteration. Stimulation of DNA repair or inhibition of cell proliferation do not seem viable prospects for clinical intervention at this time. Step 5: The conversion of initiated cells to preneoplastic cells is a lengthy process and appears to currently offer the best prospects for clinical intervention. Even a small delay in this process would result in a significant decrease in incidence. Step 6: The phenotypic reversion of preneoplastic cells to normality, thus preventing the progression to frank neoplasia, is feasible and offers the advantage of rapid assessment of drug efficiency.

Initiation. This process involves the conversion of a normal cell to one which is initiated. Initiation appears to be a permanent event but its effects are latent, requiring decades in humans, months in experimental rodents, and weeks in cell culture models to become evident as the phenotypic change we call cancer. The permanency and latency in man is perhaps best illustrated by the continued development of excess malignancy among the survivors of the Japanese atomic bombings. As currently understood, initiation involves the chemical reaction of an activated carcinogen with a relevant DNA base, and the subsequent attempt by the cell to replicate this damaged DNA during normal mitogenesis. However, due to the presence of the carcinogen adduct, normal base-pairing cannot occur and a mutation is introduced to the daughter DNA. The simplest example of carcinogen activation and DNA adduct formation is the formation of a methylcarbonium ion from the widely distributed carcinogen dimethylnitrosamine, and its subsequent reaction to form a 0-6-methyl adduct on guanine. The presence of this adduct prevents normal base pairing with cytosine and makes likely the erroneous incorporation of thymidine. It is of interest that activated oncogenes of the ras family, which are frequently isolated from human carcinomas have apparently been activated by a cytosine ---> thymidine point mutation consistent with the mechanism outlined above (Tabin et al., 1982).

This production of covalent damage to DNA bases by classical chemical carcinogens, is identical in all important respects to the DNA damage produced by those cancer chemotherapeutic agents classed as alkylating agents, and with much of the damage produced by ionizing radiation. In fact, any chemotherapeutic agent which has DNA binding properties, such as cyclophosphamide, or which is erroneously incorporated into DNA, such as cytosine arabinoside (araC) can be expected to initiate a proportion of surviving cells. The development of secondary, drug-induced malignancies in individuals apparently cured by chemotherapy of their original neoplasm is a tragic consequence of the carcinogenic properties of many chemotherapeutic agents (Harris, 1976). This subject has been addressed in detail at this symposium.

Several sites exist for the prevention of initiation. Because many carcinogens require metabolic activation to their ultimate carcinogenic form which reacts with DNA (Autrup and Harris, 1983), blocking metabolic activation should prevent DNA damage. For example, carcinogens such as benzo[a]pyrene (BP), formed during incomplete combustion, have alternative routes of metabolism not resulting in activation. Flavones and butylated hydroxytoluene (BHT) can alter the metabolism of BP and protect experimental animals from carcinogenesis (Wattenberg, 1985). There is epidemiologic evidence suggesting that consumption of foods, such as cruciferous vegetables, rich in flavones and other compounds capable of altering the metabolism of BP, protect against GI cancer (Graham et al., 1972), however a protective role for these compounds in human neoplasia is quite unclear. The most persuasive evidence that carcinogen formation can be blocked comes from studies of the role of ascorbic acid in preventing the formation of carcinogenic nitrosamines in the stomach (Mirvish, 1983). Here endogeneous formation of nitrosamines from diets high in pickled and salted foods has been associated with high gastric cancer rates (Correa et al., 1975), and the declining incidence of gastric cancer can be correlated with the decreased use of such food preservation methods and the increased availability, year-round, of foods containing ascorbic acid.

In theory at least, compounds which block reaction of the proximal reactive form of a carcinogen with DNA, should be highly effective chemopreventive agents. Because most proximal carcinogens are electrophiles (Miller and Miller, 1966), any strongly nucleophilic compound can be expected to have activity. With few exceptions however, results of studies utilizing agents of this type have been disappointing when conducted in whole animals. The reason for lack of activity may imply that in most cases, metabolic activation takes place within the target cell, and that in order to be effective in blocking reaction with DNA, the compound must intercept the proximal carcinogen, probably within milliseconds of formation, and must compete with available tissue nucleophiles for covalent binding. In view of the high concentration of tissue nucleophiles and the low concentration of proximal carcinogens to be expected in human tissues, this may be an unrealistic goal.

In terms of preventing systemic carcinogenic damage resulting from cancer chemotherapeutic regimes, there is little doubt that the host can be spared. Strategies such as local infusion of highly reactive agents like mechlorethamine with its short half life, or the use of thiosulphate to protect peripheral tissues have been used. However, when chemotherapy is directed towards disseminated disease, strategies to spare the host will most probably also spare the tumor.

In summary, while prevention of initiation, which prevents the production of cellular damage, has many theoretical advantages, it has many practical limitations. Intervention must occur during exposure to a carcinogen and thus cannot be instituted retrospectively; high concentrations of chemopreventive agent may be required may be toxic; for those compounds which alter metabolism, when used in humans which are exposed to diverse carcinogens, blockage of one carcinogen may result in activation of another; finally because of the protracted nature of carcinogenesis in humans, the results of anti-initiation protocols may take decades to evaluate.

Inhibition of the Promotional Phase of Carcinogenesis

Promotion and Promoters. This phase is the longest, the least understood, and yet is the phase with the greatest potential for clinical intervention. This potential stems from several separate lines of evidence: 1) consequences of exposure to chemicals and radiation demonstrate that this phase is of many years duration in humans. It is possible, therefore, unlike anti-initiation therapy, to begin treatment long after commencement of carcinogen exposure; 2) since late stages of carcinogenesis are affected it should be possible to detect results in a few years rather than the decades required to demonstrate effectiveness of anti-initiation therapy; 3) from the results of animal studies, and from epidemiologic evidence, drugs are available which demonstrate activity without appreciable side effects; 4) experience in both humans and animals has shown early preneoplastic lesions can be reversed. A potential disadvantage of certain drugs active in this phase is that studies in both animals and in cell culture show that inhibition of promotion by retinoids, the compounds currently showing greatest promise, is reversible, requiring continuous treatment for continuous effect. Thus, should clinical efficacy be unequivocally demonstrated, the ethics of discontinuing therapy in clinical trial volunteers will require careful consideration.

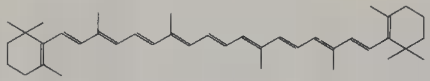
Intervention with Retinoids and Carotenoids. Because of the extensive evidence for the role of retinoids as inhibitors of carcinogenesis in experimental studies, as chemopreventive agents in recent clinical trials, and because of the interest of my group in this group of compounds, this discussion will focus exclusively on these compounds. For a more extensive review of this area, see Bertram, et al., 1987 and Moon and Itri, 1984. Retinoids are defined as natural synthetic derivatives of vitamin A which are able to support normal growth and differentiation (but not necessarily vision). Carotenoids are polyprenes of plant origin which may or may not have pro-vitamin A activity.

Epidemiologic studies evidence for the protective role of carotenoids and/or retinoids in cancer is persuasive. Several studies have demonstrated a reduced risk of carcinoma of the lung, oral cavity, esophagus, stomach and bladder (reviewed in Bertram et al., 1987). In general, the associations have been stronger for carotenoids than retinoids, however, it should be noted that because of the conversion of many carotenoids to retinoids in the intestine and liver, it is difficult to separate the individual contributions of these compounds. Furthermore, while many studies did not attempt to distinguish these individual contributions, others evaluated only the the β-carotene component of dietary intake; other carotenoids may, as discussed later, have greater biological activities.

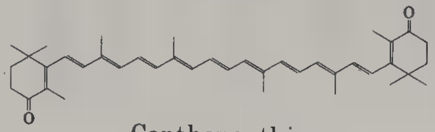
Experimental studies in animals have more clearly demonstrated the protective role of retinoids against chemical carcinogen-induced carcinomas of the skin, mammary gland, bladder, oral cavity, esophagus and fore-stomach (reviewed in Bertram et al., 1987). Both natural and synthetic retinoids can reduce the rate of cancer induction at many sites when applied after the carcinogen (i.e. in the promotional phase of carcinogenesis). Carotenoids have been less extensively studied in vivo, and because of their poor absorption and extensive metabolism by rodents, no good animal model exists. Nevertheless, it is clear that carotenoids will protect against UV-induced skin tumors (Epstein, 1977).

My group has made extensive use of the C3H/10T1/2 (10T1/2) mouse fibroblast system (Reznikoff et al., 1973) to examine activities of retinoids and carotenoids against chemically- and physicially-induced neoplastic transformation. This system mirrors in remarkable detail events known to occur in animals and man (Bertram, 1985) and can be accurately quantitated. It has the additional specific advantage that these cells do not convert carotenoids to retinoids (Rundhaug et al., 1988), allowing the separate analysis of these compounds. Work with these cells has clearly demonstrated that retinoids act in the post-initiation phase of carcinogenesis. Indeed treatment is still effective when delayed until just prior to the first appearance of transformed cells, some 3-4 weeks after removal of the carcinogen. Moreover, inhibition of formation of transformed foci of cells, the endpoint of the assay, is achieved at concentrations of retinoids without cytotoxicity to either the normal or transformed cells. This lack of toxicity was confirmed by the observation that upon withdrawal of retinoids from carcinogen-treated cultures, transformed foci appeared in numbers comparable to those in carcinogen-only treated controls (Merriman and Bertram, 1979). These results led us to propose that retinoids act by stabilizing the initiated state of the cell but not eliminating its neoplastic potential. The reversible nature of retinoid action has since been confirmed in an animal study (Thompson et al., 1979), and has important implications to the clinic. While demonstrating that retinoids act long after carcinogen exposure, a desirable attribute in any high risk group identified because of prior exposure (e.g. tobacco users), the potential reversibility is disturbing as it implies that continued intervention is required for continued prevention.

Recent studies have evaluated the activity of carotenoids in the 10T1/2 system with the goal of determining if these compounds have intrinsic activities as chemopreventive agents as suggested by the epidemiologic data. The effects of β-carotene (β-C), the carotenoid with the highest pro-vitamin A activity, were compared with the effects of canthaxanthin, a closely related molecule which lacks activity (Figure 2).



β-Carotene



Canthaxanthin

Figure 2.

Compounds were administered in an oil-in-water emulsion, which itself did not influence the assay but allowed efficient uptake by the cells. This is the same formulation as currently used in clinical trials of β-C.

When these carotenoids were added 7 d after removal of a transforming dose of methylcholanthrene (MCA), and maintained throughout the 4 week remainder of the assay period, they were found to induce a profound dose-dependent reduction in the formation of transformed foci (Figure 3). Across the entire dose range canthaxanthin was more potent than β-C with ED_{50} concentrations of 2 x 10^{-7} and 10^{-6} M respectively. Upon removal of carotenoids from carcinogen-treated cultures after a 4 week treatment period, foci appeared after a 2-5 week latent period (Figure 4).

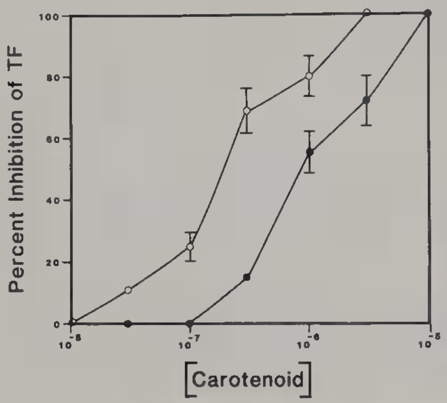


Figure 3. Effects of carotenoids on neoplastic transformation of 10T1/2 cells. β-carotene (●-●) or canthaxanthin (o-o) were added 7 days after treatment of 10T1/2 cells with methylcholanthrene and maintained in cultures by weekly re-treatment. After 4 weeks, cultures were fixed and stained, the number of transformed foci quantitated and the transformation frequency (TF) calculated. For experimental details, see (Pung et al., 1988).

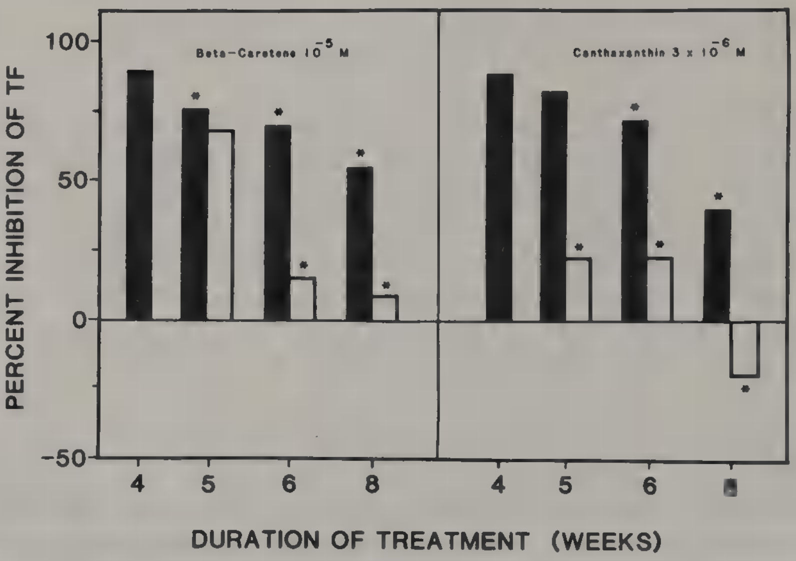


Figure 4. Effects of removal of carotenoids from carcinogen-treated cultures. After 4 weeks of carotenoid treatment as in Figure 3 replicate cultures were either maintained with carotenoid, or had treatment withdrawn. The development of transformed foci was quantitated with time. Solid bars, continuous treatment; open bars, treatment withdrawn. (For experimental details see Pung et al., 1988)

In other studies using X-rays as initiator (500 rad) both carotenoids were found to be ineffective when added prior to and during irradiation, but, as with chemical carcinogens, were effective when added during the post-initiation period (Pung et al., 1988). These results are remarkably similar to those obtained with retinoids (Merriman and Bertram, 1979), yet it should be remembered, carotenoids have no known activity as vitamin A analogs prior to their biological conversion.

To investigate if 10T1/2 cells were capable of converting β-C to retinoids, ^{14}C-β-C, [illegible] gift from Hofmann-LaRoche, was added to confluent cultures for 1 or 7 days. Analysis of culture medium and of cells by high-pressure liquid chromatography demonstrated extensive cellular uptake of β-C, but failed to reveal any excess ^{14}C associated with peaks of non-radioactive retinoids corresponding to the expected products of β-C metabolism (i.e., retinal, retinol and retinoic acid).

We thus concluded that β-C has intrinsic activity as a cancer preventive agent (Rundhaug et al., 1988).

Clinical Studies. Based upon the epidemiologic and the consistent experimental evidence outlined above, a number of clinical trials of carotenoids and retinoids have been conducted in individuals at high risk to cancer. A summary of some major trials is presented in Table 1.

Table 1. Selected Chemoprevention Trials

Target Site	Retinoid	Outcome	Ref.
Bladder	Etretinate	↓ Recurrence Rate	22
Cervix	Trans-retinoic acid	↓ Dysplasia	23,24
Bronchus	Etretinate	↓ Metaplasia	25
Oral Cavity	Trans- or 13-cis Retinoic Acid	↓ Leukoplakia	26,27
Skin	Trans-Retinoic Acid	↓ Actinic Keratosis	28
Skin	13-cis Retinoic Acid	↓ Basal Cell Carcinoma	29

An important observation is that both natural and synthetic retinoids have been demonstrated to cause the regression of pre-neoplasia in a number of organ sites. These sites were originally chosen to be accessible and thus easily monitored, and there seems little doubt that other sites will also be amenable to such interventional therapy. For a more detailed discussion of these results, the special problems posed by chemoprevention trials and a list of more than 20 on-going clinical trials, see Bertram et al., 1987. Most of these trials involve the use of retinoids or carotenoids. An interesting observation in several of these completed trials is that the activity of synthetic retinoids extends to basal and squamous carcinomas of the skin, indicating that in this organ at least, retinoids are active beyond the pre-neoplastic stage.

The mechanism of action of retinoids is currently unknown, except for the role of retinal in vision. Based upon our work in 10T1/2 cells, we have developed the hypothesis that in this system, retinoids suppress transformation by enhancing the transfer of growth control signals via intercellular gap junctional communication. This hypothesis is based upon: 1) the known ability of normal cells to suppress the growth of transformed cells (Bertram and Faletto, 1985) and the strong correlation between this suppression and junctional communication between these two cell types (Mehta et al., 1986); 2) the ability of retinoids to up-regulate junctional communication in normal cells and in transformed cells (Mehta et al., 1989) or in carcinogen-initiated cells (Hossain et al., submitted). This model is consistent with the action of retinoids on the post-initiation phase of carcinogenesis and with the reversible nature of this action. Further research is required to determined if this model can be extended to human cells, particularly those of epithelial origin.

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RETINOIDS AND BREAST CANCER

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INTRODUCTION

The anti-proliferative and cell differentiation properties of retinoids are at the basis of their use in chemoprevention which may be envisioned under two headings: firstly, to prevent tumor occurrence by inducing premalignant cells to differentiate, thereby arresting their progression or causing their reversal. Secondly, to prevent tumor recurrence or relapse by inducing clonogenic tumor cells to differentiate. Considering the latter, it is clear that any increase in clonogenic tumor cell differentiation will result in tumor growth curve modification depending on whether the rate of differentiation (d) is less, equal to or greater than the birth rate (b) of these cells. For d less than b, the growth curve will continue to increase but at a slower rate; when d equals b, the growth rate will level off, and if d exceeds b, regression will occur. Hence, retinoids could be of particular value in a combined modality approach to enhance the effect of chemotherapy by driving residual clonogenic cells into the transitional cell compartment.

Strictly speaking, however, chemoprevention refers to intervention measures during the development of precursor lesions and it is in this context that the topic will be further discussed. In particular, the issue of dose in relation to the stages of tumor formation will be specifically addressed based on an overview of experimental, epidemiologic and clinical evidence.

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Before doing so, it will be useful, in order to exemplify how epidemiologic information may be integrated to chemoprevention strategies, to first consider the role of epidemiology in estimating the probability of developing breast cancer to identify, in a given population, subjects eligible for a chemoprevention trial.

SELECTION OF A HIGH RISK TARGET POPULATION FOR CHEMOPREVENTION

An individual's probability of developing breast cancer can be calculated if the following information is known: 1) the woman's risk factor or combination of risk factors; 2) the relative risk for each factor; 3) the breast cancer incidence rate in the population; 4) the distribution of risk factors in the population. Epidemiologic studies have revealed a number of well known risk factors for breast cancer (table 1) which, except for pathological and radiologic data, may be obtained from the women herself.

TABLE 1: Breast Cancer Risk Factors

- Age
- Age at menarche
- Age at first full-term pregnancy
- Age at menopause
- Nulliparity
- Family history of breast cancer in a first degree relative
- Biopsied benign breast disease
- Epithelial hyperplasia with or without atypia
- P_2 and/or Dy mammographic parenchymal patterns

Similarly, relative risk estimates for each risk factor may easily be found in the literature and range from about 2 for nulliparity to 5 for a finding of atypical epithelial hyperplasia at biopsy. The reader is referred to reviews on the subject for detailed information (Boyd et al., 1982; Dupont and Page, 1985; Henderson et al., 1984; Kelsey and Hildreth, 1983; MacMahon et al., 1972; Page et al., 1985; Saftlas and Szklo, 1987). Incidence data may be derived from a population-based tumor registry such as the British Columbia Cancer Registry. For instance, in the age group 50 to 59 years, the incidence of breast cancer is about 200 per 100000 (table 2).

TABLE 2: Age-specific Breast Cancer Incidence Rates for British Columbia in 1987.

Age group (years)	Annual incidence rate per 10^5 women
<30	1
30-39	37
40-49	126
50-59	185
60-69	340
70-79	355
80+	366

In contrast, information on the distribution of risk factors in the population is not commonly available. In the example to follow, we have assessed the distribution of breast cancer risk factors in 1118 women aged 50 to 59 years attending the Canadian National Breast Cancer Screening Study in Vancouver (Miller et al., 1981). Four known breast cancer risk factors were considered (table 3): nulliparity (N); age at first birth at 30 years or over (A); family history of breast cancer in a first degree relative (F); two or more biopsies for benign breast disease (B).

TABLE 3: Breast Cancer Risk Factors in 1118 Women Aged 50 to 59 Years attending the Canadian National Breast Screening Study in Vancouver.

Risk Factor*	Proportion of Population	Relative Risk
0	63.3	1.0
N	10.1	2.0
A	9.1	2.0
F	10.4	2.5
B	3.3	3.0
FN	1.0	5.0
FA	1.5	5.0
BN	0.6	6.0
BA	0.1	6.0
FB	0.4	7.5
FBN	0.1	15.0
FBA	0.2	15.0

Mean population relative risk: 1.6

* 0: absence of risk factor; N: nulliparity; A: age at first birth at 30 years or over; F: family history of breast cancer in a first degree relative; B: 2 or more biopsies for benign breast disease.

Relative risks for individual risk factors were obtained from the literature and it was assumed that the relative risk for multiple factors is the joint product of the relative risk for each factor. Based on the data shown in table 3, the cumulative proportion of women with specific mean relative risk can be calculated. For example the 17.6% women who have a relative risk of 2.5 or greater (table 3) have a *mean* relative risk of 3.4. Similarly, the 1.4% who have a relative risk of 6 or greater (table 3) have a *mean* relative risk of 8.4 (table 4).

TABLE 4: Cumulative Proportion of Women Aged 50 to 59 Years with Indicated Mean Relative Risk using the Risk Factors Shown in Table 3.

Mean Relative Risk	Cumulative Proportion (%)
15.0	0.3
10.7	0.7
10.1	0.8
8.4	1.4
6.6	2.9
6.2	3.9
4.7	7.2
3.4	17.6
3.0	27.1
2.7	36.7
1.6	100.0

Entries were calculated by combining all groups with the same or higher relative risk (table 3) and calculating the relative risk of the resulting group as the weighted sum of the individual risk of the component groups.

With the information at hand, the proportion of women eligible for a chemoprevention trial may be determined given that the probability of developing the disease over the trial duration is specified. In the example displayed in table 5, for a 5 year trial in women aged 50-59 years where a 2% probability of developing breast cancer is required, the mean relative risk of the group would be 3.2 and 17.6% of women would be eligible. Similarly, for a 5% probability of developing disease, the figures would be 8.1 and 1.4% respectively.

TABLE 5: Proportion of Women Aged 50 to 59 Years Eligible for a Chemoprevention Trial of 5 Year Duration.

Breast Cancer Probability (%)	Mean Relative Risk Required	Proportion Eligible (%)
2	3.2	17.6
3	4.9	3.9
4	6.5	2.9
5	8.1	1.4

When planning a randomized chemoprevention trial for breast cancer, sample size is of critical concern. As an example, let us consider a two-arm trial in which one is a placebo arm, and the hypothesized reduction in breast cancer risk due to chemoprevention ranges from 25% to 50%. For a group with a 2% probability of developing breast cancer over a fixed time interval of 5 years, and for a relative risk reduction of 25%, a total of 17800 participants would be required (table 6).

TABLE 6: Total Sample Size for a Two-arm Chemopreventive Trial of 5 Year Duration in Women Aged 50 to 59 Years.

Relative Risk Reduction (%)	Specified Disease Probability	
	2%	5%
	Sample Size	Sample Size
25	17,800	6,940
30	12,140	4,740
40	6,580	2,580
50	4,040	1,600

P = .05
Power = .80

A similar trial in a group with a 5% probability of breast cancer over the study period and for the same reduction in relative risk would need 6940 subjects. Clearly, sample size varies inversely with the anticipated relative risk reduction and with the specified probability of disease occurrence. However, as the specified disease probability is increased, so does the required relative risk with the result that the proportion of eligible participants dramatically decreases (table 5).

CHEMOPREVENTION

Carcinogenesis is a complex multi-step process evolving over a prolonged period of time, which has been characterized into the stages of initiation, promotion and progression (Berenblum, 1982; Farber, 1984). Initiation results in hereditary and irreversible cellular genetic damage induced by carcinogens. Promotion leads to clonal expansion of the initiated cells and to a variety of phenotypic changes which may become irreversible over time (Weinstein et al, 1979). The poorly understood process of progression encompasses the subsequent development of precursor lesions and cancer.

When considering chemopreventive strategies at least two phases of the neoplastic process must conceptually be distinguished. Firstly, the initiation and early reversible promotion phase; secondly, the later more autonomous phase of tumor development. We suggest that these distinctions are of crucial importance as the effective dose of chemopreventive agents may differ depending on the stage of tumor development. Failure to take these considerations into account could result in negative or inconclusive chemoprevention trials. This discussion focusses on retinoids (vitamin A and its synthetic analogs) since these substances have been the most thoroughly studied for their chemopreventive properties in vitro and in vivo and are currently undergoing intensive clinical trial (De Wys and Greenwald, 1983). For simplicity the term retinoids will be used in this text for both vitamin A and its derivatives unless otherwise specified.

Anti-Carcinogenic Action of Retinoids

Retinoids suppress, in vitro, the process of malignant transformation (Chatterjee and Banerjee, 1982; Harisiadis et al., 1978; Merriman and Bertram, 1979; Todaro et al., 1978), restore contact inhibition and anchorage-dependent growth to transformed cells (Dion et al., 1977; Dion et al., 1978), and inhibit or reverse the hyperplastic and metaplastic changes produced by carcinogens in organ cultures of various organs including the mammary gland (Crocker and Sanders, 1970; Dickens et al., 1979; Lane and Miller, 1976; Lasnitzki, 1955; Lasnitski, 1976). In vivo, many studies have similarly shown that retinoids inhibit the process of carcinogenesis in many organs (Bollag, 1972; Bollag, 1974; Saffiotti et al., 1967; Sporn et al., 1977), notably in the mammary gland (Grubbs et al., 1977; McCormick and Moon, 1982; McCormick and Moon, 1986; McCormick et al., 1980; McCormick et al., 1983; Mehta et al., 1983; Thompson et al., 1982; Welsch et al., 1980; Welsh et al., 1984).

Although not fully elucidated, the mechanism by which retinoids exert their anti-carcinogenic action appears to be mainly related to their anti-promotion effect (Bollag, 1972; Verma and Boutwell, 1977) and to their role in controlling cellular proliferation (Fraker et al., 1984; Moon et al., 1979; Wetherall and Taylor, 1986) and differentiation. Of particular significance is the ability of vitamin A and retinoids to enhance differentiation of putative premalignant lesions (Lasnitzki, 1955; Lasnitzki, 1976) and of fully transformed malignant cells (Breitman et al., 1980; Strickland and Sawey, 1980). These effects imply a role of vitamin A in the control of gene expression involved in cell differentiation (Sporn and Roberts, 1983), possibly through a direct interaction with nuclear DNA mediated by specific intracellular retinoid-binding proteins (Jetten and Jetten, 1979). Retinoic acid binding proteins have been documented in human breast dysplastic and neoplastic cells (Huber et al., 1978; Kung et al., 1980).

Distinction Between Maintenance of Normal Cellular Differentiation and Chemoprevention

Vitamin A is essential for the maintenance of normal epithelial differentiation. Vitamin A deficiency leads in many tissues to squamous cell metaplasia (Wolbach and Howe, 1925) morphologically similar to changes induced by chemical carcinogens (Lasnitzki, 1962), and to an increased susceptibility to tumor formation (Cohen et al., 1976; Fujimaki, 1926; Nettesheim et al., 1979; Rogers et al., 1973). Of special interest are the observations that this increased susceptibility is prevented by normal or moderately increased dietary levels of vitamin A, with no added protective effect provided by high levels of this substance in the diet (Cohen et al., 1976; Fujimaki, 1926; Nettesheim et al., 1979; Rogers et al., 1973;). In parallel, a number of independent epidemiologic studies have shown an inverse correlation between the frequent consumption of food rich in vitamin A and/or provitamin A (beta-carotene) and cancer risk (Kummet et al., 1983; Peto et al, 1981). These studies, which substantiate experimental data, indicate that adequate or moderately increased dietary levels of vitamin A and/or its provitamin, exert a protective effect against cancer development in humans. Taken together, the experimental and epidemiologic evidence point to the effectiveness of maintenance levels of vitamin A, and/or provitamin A, in inhibiting the initiation and/or reversible promotion processes. As preneoplastic lesions develop, however, it is clear that this protective effect has been overwhelmed.

In vitro studies aimed at reversing established precursor lesions induced by chemical carcinogens have either required high doses of vitamin A (Lasnitzki, 1955) or shown a dose-dependent (Lasnitzki, 1976) or a time-dependent effect with decreasing effectiveness related to delayed retinoid administration (McCormick and Moon, 1982). In experimental animal systems pharmacologic doses of retinoids have generally been necessary to inhibit tumor development (Crocker and Sanders, 1970; Grubbs et al., 1977; Thompson et al., 1981). Retinoids given in the post-initiation phase of the carcinogenesis process have also shown a dose-dependent inhibition of preneoplastic and malignant lesions (Bollag, 1974; Moon et al., 1979; Sporn et al., 1977; Thompson et al., 1981), and a progressive loss of chemopreventive potential associated with delayed retinoid treatment (McCormick and Moon, 1982). Furthermore, whereas low non-toxic doses of retinoids are effective in inducing differentiation of malignant cells in vitro (Breitman et al., 1980; Strickland and Sawey, 1980), high toxic doses have been required to produce similar changes in vivo (Strickland and Sawey, 1980).

In humans, retinoids have shown promising chemopreventive activity in a variety of premalignant conditions (Gouveia et al., 1982; Hong et al., 1986; Kerndrup et al., 1986), in patients with high risk benign breast disease (Band et al., 1984), and in preventing tumor recurrence (Alfthan et al., 1983; Peck et al., 1982). These clinical trials, which generally used a single dosage-schedule, have required the administration of pharmacologic doses of retinoids invariably associated with toxicity except in one study (Gouveia et al., 1982). Since dose-response effects were not evaluated, it is impossible to conclude whether lower non-toxic doses would have been active. However, a reduction in retinoid dosage has been correlated with a decreased therapeutic activity (Koch, 1978; Peck et al., 1982), and in a large chemoprevention trial in patients with premalignant esophageal lesions, doses of vitamin A of about two to three times the recommended daily allowance have been shown to be ineffective (Munoz et al., 1985).

It would thus appear that the effective doses of retinoids depend on the timing of their administration in relation to the stage of cancer development. Considering the protective effect of early and long term administration of maintenance levels of vitamin A and/or beta-carotene against cancer formation, it would seem justified to recommend an adequate dietary intake of food rich in these substances to reduce cancer incidence in the general population.

In the more advanced stages of tumor development, particularly in the presence of premalignant lesions, maintenance levels of vitamin A or equivalent doses of retinoids are likely to be ineffective and pharmacologic treatment with these compounds in needed. Since currently used retinoids produce dose limiting side effects (Alfthan et al., 1983; Band et al., 1982; Bollag, 1983), a certain degree of toxicity will be difficult to avoid and will need to be accepted until new synthetic compounds with improved therapeutic indices become clinically available.

CONCLUSION

The efficacy of retinoids against mammary gland tumor development has been amply documented experimentally, setting the stage for chemoprevention trials in high risk women. However, several problems confront the planning of chemoprevention trials of breast cancer. Firstly, in comparison to smoking as a risk for lung cancer, the individual risk factors for breast cancer are generally weak. Consequently, selection of participants needs to be based on combinations of risk factors satisfying the minimum level of risk required by the trial. Setting a high risk level reduces the number of study participants required but also reduces the number of eligible candidates in the population. Hence, specification of the risk level for participant selection is critically dependent on the knowledge of the distribution of risk factors in the population where the study is contemplated. It is likely that the high risk mammographic patterns will play an increasing role in the selection of women for intervention trials, in situations where the population distribution of these patterns is known.

Secondly, whereas chemoprevention induced reversal of premalignant lesions of the cervix could readily be noted by repeated cytologies, the possibility of observing similar changes in the breast would be difficult to obtain. Thus, the potential efficacy of an agent for breast cancer chemoprevention must be inferred from evidence of activity in animal mammary tumor models and possibly, in humans, from reversal of dysplasia observed in sites other than the breast.

Retinoids and chemopreventive agents in general ought to be investigated in a manner analogous to that of chemotherapeutic drugs. Thus, phase I pharmacologic and toxicologic studies should be undertaken to determine pharmacokinetic parameters, maximum tolerated dose, toxicity, and the time-course pattern of the reversibility of side effects. Prior to considering large intervention trials, phase II studies need to be carried out in patients having well defined precursor lesions such as dysplastic oral leukoplakia, cervical dysplasia, or actinic keratosis, with particular attention to dose-response effect. In this respect, the dose-intensity approach recently suggested for the investigation of chemotherapeutic drugs (Hryniuk and Bush, 1984) appears equally appropriate to phase II studies aimed at establishing the dose-response curves of chemopreventive agents. Careful monitoring of the lesions would enable to determine the dose range at which regressions are observed, the degree and duration of regression, as well as an eventual enhancement effect (Schroder and Bloch, 1980). Furthermore, such studies would enable to select the least toxic effective chemopreventive dose for intervention trials in high risk populations which could then be undertaken based on evidence of activity.

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COMMENTS ON FUTURE DEVELOPMENTS OF CANCER THERAPY TAKING INTO CONSIDERATION TUMOR BIOLOGY AND KINETICS. PART I.

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INTRODUCTION

This volume contains the presentations of the International Symposium on Effects of Therapy on Biology and Kinetics of Surviving Tumor, held in Spring of 1989 in Vancouver, Canada. Several important aspects of therapy, including its benefits and side effects as well as the introduction of new treatment modalities are comprehensively reviewed. It has been stated by the members of the symposium scientific committee, that "one of the best projections for the future of cancer therapy is to imagine a successful termination of most of the discussed projects presented in this volume". Such statement may be close to truth, as most of the ideas and trials discussed here represent the main developments in the field of cancer therapy. Summary comments regarding future developments of these key issues are discussed below.

KINETICS AND TREATMENT PLANNING

Several papers in this volume show that important kinetic alterations take place as a result of therapeutic intervention, particularly after non-curative cytoreductive procedures. The task of future investigations will be to conclusively document if such phenomena also occur in the clinical setting. If documented, the next important step will be to assess the degree to which they adversely, or positively affect tumor behaviour. Also of major importance is the development of new therapeutic strategies to prevent or assist these phenomena.

The therapeutic approaches expected to be of benefit in this regard are, for instance, preoperative treatment amd a wider utilization of needle aspirations replacing open biopsies as the primary diagnostic technique. These approaches are already being tested by trials such as those organized in North America by the Vancouver, and more recently by NSABP groups. Other treatment approaches may include reduction, or even elimination of major surgery. This would be possible only if alternative, effective systemic and/or locoregional treatment were available, such as practised already under the auspicies of "neoadjuvant treatment" at the Salpetierre Hospital in Paris.

Other future approaches concerning tumor kinetics consist of the development of more effective scheduling and dose intensive therapies to overcome resistance, as discussed in the second volume. Most importantly, the introduction of new therapeutic approaches such as inactivation of growth factors, utilization of differentiating agents rendering tumors less aggressive, or using effective genetic manipulations to switch off genes implicated in tumor propagation, portends a rapid improvement in cancer care.

GROWTH FACTORS AND GENETIC CONTROL OF TUMOR GROWTH

1.) Growth Factors

This volume provides some evidence that identified gene products - growth factors - produced either by the tumor or host, the overall tumor growth. While multiple growth factors have already been already purified, it is believed that only a small fraction of them is presently known, and in the near future, many more may be isolated. It is likely that two aspects relating to growth factors will be of significant importance.

a.) Firstly, it is expected, though not yet proven, that growth factors may be involved in the first stages of malignant transformation. If shown to be true, their isolation or identification within high risk, non-malignant lesions may become an effective screening method to identify high risk populations.

b.) Secondly, inactivation of growth factors, peripherally with monoclonal antibodies, or via suppression of responsible genes, may be of a significant therapeutic value surpassing in its refinement the presently utilized, more crude therapeutic cancer modalities. The diagnostic and therapeutic aspects of growth factors as well as their genetic manipulation, therefore, remain one the most promising developments for future cancer research.

2.) Genetic Control of Tumor Growth

It is now evident that genes play a fundamental role in tumor etiology. They may be responsible for determining both the propensity of a benign tissue to undergo malignant transformation, as well as the phenotypic characteristics of established malignancy.

Several papers of this volume point out the fact that genetic research is already of practical significance. Oncogenes have been successfully associated with individual malignancies. In some tumors, the presence of oncogenes, and particularly their amplification and identification of overexpressed product, is correlated with tumor aggressiveness. If confirmed, such an assessment may soon aid in the refinement of staging and risk assessment at diagnosis. Also, identification of chromosomal changes and abnormal chromosomes,such as seen in some forms of leukemias, may pin-point the DNA loci, where a likely oncogenic or other significant DNA aberration may reside. The following applications of oncogenic techniques are projected to of significant assistance in the near future.

a.) Role of oncogenes in screening of high risk population

There are indications that within individuals risk groups (increased family history of cancer, smokers, nulliparas, asbestos or uranium workers, etc), individuals at risk may with identified by the aid of oncogenes or their products. This approach may result in population - wide screening, where the white blood cells, or more directly, tissues of the targeted organs, will be analyzed.

b.) Distinction of invasive from in-situ or precancerous lesions

Several oncogenes have been found present or associated with invasive human malignancies. The best examples are n-myc in neuroblastoma, c-abl in CML, H-ras in bladder, breast or lung cancers, N-ras in AML and more recently in ALL, Ki-ras in lung and G.I. malignancies, c-myc in Burkitt's lymphoma, bcll and bcl2 in lymphomas, etc. At times, histological identification of invasiveness is not clear, and importantly, not all premalignant lesions may evolve into invasive cancers. Presence of amplified oncogenes or their products in these conditions may connote invasiveness (e.g. n-myc oncogene in neuroblastoma, c-ras oncogene in breast cancer, or n-ras oncogene in myelodysplasia), and may also predict earlier, which premalignant lesions are to become malignant. Therefore, such an assessment may have direct diagnostic and therapeutic implications.

c.) Risk assessment of established malignancies

There are data suggesting that expressed or amplified oncogenes may assist in distinguishing high risk aggressive versus non-aggressive lesions at the time of initial diagnosis. Such examples are already seen in the case of n-myc oncogene in neuroblastoma, or c-HER/NEU or H-ras oncogenes in breast carcinoma. The significance of these observations cannot be underestimated, as treatment of different intensities, durations and schedules, may be indicated for lesions of different risk.

d.) Therapeutic implications of oncogene research

There are indications that the identification of oncogenes and their association with the regulation of tumor growth, including the production of stimulatory or inhibitory growth factors and their receptors, may permit their inactivation through such techniques as monoclonal antibodies. Alternatively, enhancement of activity of suppressor genes (anti-oncogenes) may also result in suppression of mitogenic products, with a limitless potential for not only therapy of established tumors, but also for interventional treatments against premalignant lesions offered to individuals from high risk populations.

IMMUNITY AND METASTASES

One of the main characteristics of newly transformed malignant cells is their tendency to become more antigenic. This characteristic may permit, in the near future, more effective diagnostic identification and characterization of newly transformed clones, by techniques such as monoclonal antibodies. Emergency of new antigenes may also be responsible for their identification and elimination by the host's immunity, particularly if aided by modalities such as biological response modifiers (BRM). Activated monocytes, either with chemotherapeutic materials such as LMD (as discussed by Phillips in this volume) or by biological substances such as IL-1 or interferons, will likely show some beneficial therapeutic results in future clinical trials. The research of biological response modifiers, discussed in more detail in the second volume, show extremely promising results of many human tumors, and point to the significance of immunity in cancer control.

The presence of micrometastases at the time of initial surgery is the key factor determining the cure rate. Effective manipulation of metastases, as discussed by Price in this volume, and the recognition of their biological characteristics, including the propensity to invade, the survival in the circulation and distant sites, as well as organ specifity and rules determining the homing to different sites, are all essential elements of the science of cancer and its therapy.

DIFFERENTIATION AND CHEMOPREVENTION

Other exciting aspects of the biology and therapy of cancer is the recognition of the effects of stroma and the tumor microenvironment on the behavior of tumor, as well as research into differentiation and chemoprevention. Differentiation is a well known physiological process resulting in such basic evolutionary processes as the transition of primitive embryonal tissues into end stage functional organs. The complex process of the transition of primitive tissues into differentiated organs will have likely systemic mediators. As discussed by Parchment in this volume, similar processes but in opposite directions, may take place within de-differentiation, as a part of the formation of malignant tumors.

It is anticipated, that future research will confirm the presence of the mediators of differentiation, and prove the possible impact of their absence in the initial stages of cancer formation. Their activation by therapeutic manipulations - a term presently termed as induction of differentiation - such as with chemopreventive agents or with some conventionally available chemotherapeutic agents - may be of major therapeutic benefit. Such results if positive, may lead to a possible reduction, or even a reversal, of established metastatic potential. This will have the most desired outcome of preventing the formation of malignancy. From this point of view, differentiating agents may gain major importance as the main cancer therapeutic or preventive modalities.

STROMA AND TUMOR MICROENVIRONMENT ARE NOW KNOWN TO PLAY AN ACTIVE ROLE AFFECTING TUMOR BIOLOGY

According to most investigators active in this field, the stroma and the tumor microenvironment represent an inherent part of tumor biology. The tumor, its stroma and extracellular matrix are not to be considered separately. They are a source of multiple biological factors, some of which are acting as stimulants, some are inhibitors or differentiating substances. These biological factors also include microvasculature which regulates blood and oxygen delivery to the tumors, determining thereby the most essential metabolic and biochemical processes of tumor. More thorough recognition of these phenomena, and their therapeutic manipulation as discussed by several speakers of this session, will bear fruitful results in the control of tumor growth.

CONCLUSION

It would be reasonable to indicate in summary that integration of our knowledge of kinetics with treatment planning, utilization of data accumulated from research on areas such as resistance, genetics, immunology, radiobiology, dose intensity and support therapies, may all result in a significant improvement of treatment schedules, leading ultimately to improved cure rates of most malignancies. The recognition of genetic control of tumor growth, and the mediation of tumor growth by substances such as growth factors or differentiating biological materials will have virtually unlimited therapeutic potential. It will also allow preventive interventions in the pre-malignant stage, both for diagnostic as well as therapeutic purposes directed at high risk populations.

It is the hope of the editors, that this volume will contribute towards our understanding of the present state of the art of these most exciting areas of research.

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**EFFECTS OF THERAPY ON BIOLOGY AND KINETICS
RESIDUAL TUMOR, Part A: Pre-Clinical Aspects**

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In the management of cancer, the results of any p
approach will vary greatly, not only with a given tumor
individuals with the same tumor stage and other simil
istics. Effects of Therapy on Biology and Kinetics of t
Part A: Pre-Clinical Aspects examines the effectiveness o
modalities as applied under a range of individual condi
the behavior of residual tumors that survive such trea
pre-clinical aspects of therapy on residual tumors, this
short- and long-term consequences of various techniqu
patient as well as the carcinoma itself. This work reviews
in detail:

● Kinetics ● Kinetics and treatment design ● Growt
regulation of tumor growth ● Metastases and immunit
microenvironment, differentiation, and chemopreventio

Effects of Therapy on Biology and Kinetics of the Re
A: Pre-Clinical Aspects presents the most recent work o
authorities in the field and raises fundamental questions
anisms of tumor growth in order to point the direction
This comprehensive text will serve as both an up-to-date s
and a guide to further inquiry for researchers in carcinogenesis, oncology, and biochemistry, as well as clinicians involved in chemotherapy, radiotherapy, or surgery as means of managing cancer growth.

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